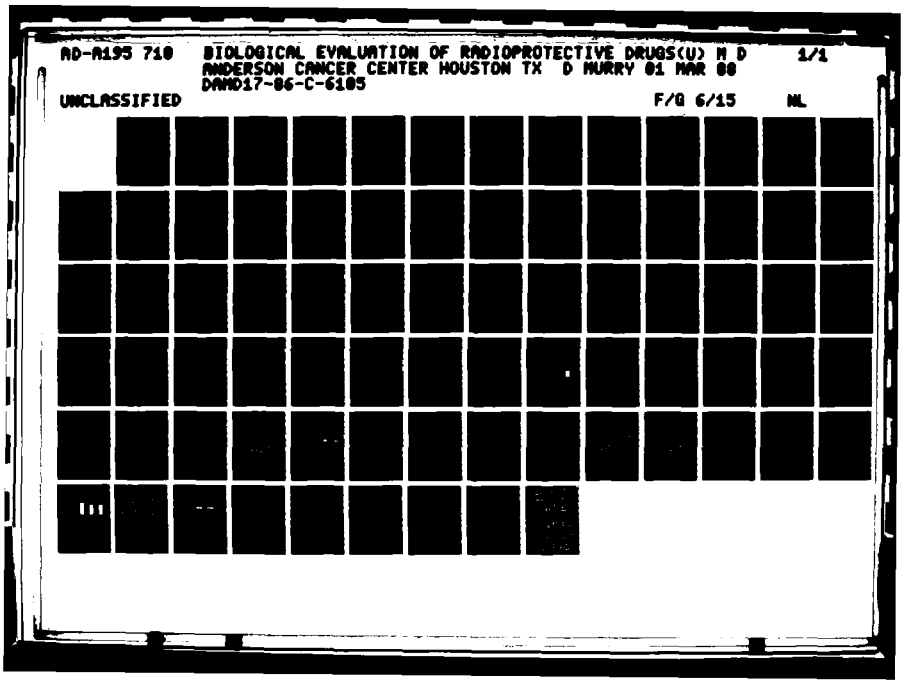
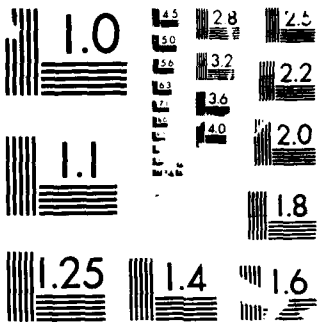

AD-A195 710 BIOLOGICAL EVALUATION OF RADIOPROTECTIVE DRUGS(U) M D 1/1
ANDERSON CANCER CENTER HOUSTON TX D MURRY 01 MAR 88
DAMD17-86-C-6105
UNCLASSIFIED F/G 6/15 NL



(2)

AD_____

BIOLOGICAL EVALUATION OF RADIOPROTECTIVE DRUGS

ANNUAL REPORT

David Murray

March 1, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6105

The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

Approved for public release; distribution unlimited

The findings in this report are not to be construed as an official Department of Army position unless so designated by other authorized documents.

AD_____

BIOLOGICAL EVALUATION OF RADIOPROTECTIVE DRUGS

ANNUAL REPORT

David Murray

March 1, 1988

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701-5012

Contract No. DAMD17-86-C-6105

The University of Texas
M.D. Anderson Cancer Center
Houston, Texas 77030

SECURITY CLASSIFICATION OF THIS PAGE

REPORT DOCUMENTATION PAGE

Form Approved OMB No. 0704-0188

1a. REPORT SECURITY CLASSIFICATION: Unclassified	1b. RESTRICTIVE MARKINGS
2a. SECURITY CLASSIFICATION AUTHORITY	3. DISTRIBUTION/AVAILABILITY OF REPORT: Approved for public release; distribution unlimited
2b. DECLASSIFICATION/DOWNGRADING SCHEDULE	
4. PERFORMING ORGANIZATION REPORT NUMBER(S)	5. MONITORING ORGANIZATION REPORT NUMBER(S)
6a. NAME OF PERFORMING ORGANIZATION: University of Texas System Cancer Center; 6b. OFFICE SYMBOL (If applicable)	7a. NAME OF MONITORING ORGANIZATION
6c. ADDRESS (City, State, and ZIP Code): Houston, Texas 77030	7b. ADDRESS (City, State, and ZIP Code)
8a. NAME OF FUNDING/SPONSORING ORGANIZATION: U.S. Army Medical Research & Development Command; 8b. OFFICE SYMBOL (If applicable)	9. PROCUREMENT INSTRUMENT IDENTIFICATION NUMBER: Contract No. DAMD17-86-C-6105
8c. ADDRESS (City, State, and ZIP Code): Fort Detrick, Frederick, Maryland 21701-5012	10. SOURCE OF FUNDING NUMBERS

PROGRAM ELEMENT NO.	PROJECT NO.	TASK NO.	WORK UNIT ACCESSION NO.
62734A	3M1-62734A875	BC	094

11. TITLE (Include Security Classification): Biological Evaluation of Radioprotective Drugs

12. PERSONAL AUTHOR(S): David Murry, Ph.D.

13a. TYPE OF REPORT	13b. TIME COVERED	14. DATE OF REPORT (Year, Month, Day)	15. PAGE COUNT
Annual Report	FROM 2/1/87 TO 1/31/88	1988 March 1	71

16. SUPPLEMENTARY NOTATION

17. COSATI CODES			18. SUBJECT TERMS (Continue on reverse if necessary and identify by block number)
FIELD	GROUP	SUB-GROUP	
06	18		radioprotection - aminothiols - cultured cells - jejunum - bone marrow - mouse - cell survival - animal survival - DNA damage
06	13		

19. ABSTRACT (Continue on reverse if necessary and identify by block number)

The cellular and molecular mechanisms of radioprotection by aminothiols are being investigated through the use of both cultured mammalian (CHO) cells and a whole-animal model, specific-pathogen free mice. For each of these systems, detailed structure-activity relationships are being compiled which relate the effects of the different drugs on various endpoints for the survival of clonogenic cells and on the induction and repair of several types of DNA lesions in these same cells. The data obtained with cultured cells are used as a means of understanding or predicting the effects on tissues. A major finding is that tissue oxygenation appears to play an important role in determining both the extent and mechanism of protection, and that understanding the interrelationships between oxygen, aminothiols and radiation sensitivity will be a crucial step in our ability to utilize such drugs effectively.

20. DISTRIBUTION/AVAILABILITY OF ABSTRACT	21. ABSTRACT SECURITY CLASSIFICATION
☐ UNCLASSIFIED/UNLIMITED ☒ SAME AS RPT. ☐ DTIC USERS	Unclassified

22a. NAME OF RESPONSIBLE INDIVIDUAL	22b TELEPHONE (Include Area Code)	22c. OFFICE SYMBOL
Mrs. Virginia M. Miller	301/663-7325	SGRD-RMI-S

DD Form 1473, JUN 86 — Previous editions are obsolete. — SECURITY CLASSIFICATION OF THIS PAGE

SUMMARY

This report summarizes our second year's progress on studies of the mechanism(s) of action of aminothiol radioprotective compounds. Using cultured mammalian cells we have been accumulating detailed structure-activity relationships for a series of thiols with respect to their relative effects on cell survival, DNA single-strand break (SSB) induction and repair, and DNA double-strand break (DSB) induction and repair. Because of the considerable current interest in the measurement of DSBs we have been studying this aspect of their activity in some detail, and in particular the effect of varying the pH conditions of the neutral elution assay on the results obtained. Five compounds have now been characterized using these assays: dithiothreitol, cysteamine, WR-1065, WR-255591 and WR-151326. Based on these data we are now beginning to be able to make some conclusions about structural features and protective mechanism(s). The most intriguing observation is that the molecular processes underlying radioprotection vary markedly with the changes in thiol structure, suggesting in turn that there is no single "mechanism" of protection. Each of the drugs appears to have a differential effect on the induction of different classes of DNA lesion, and this behavior varies among the drugs. Studies are also being performed to evaluate the relationships between DNA SSB induction, clonogenic stem-cell survival, and loss of tissue function in several different mouse tissues irradiated in vivo. Thus far we have characterized two compounds in detail--WR-2721 and WR-3689--for their effects on DNA damage and clonogenic cell survival in bone marrow and jejunum. The effects on clonogenic cell survival correlated closely with the effects of the drugs on the survival of the animals. Again, the results with the DNA damage assay are intriguing and suggest an important role for oxygen in modifying the relationships between DNA-level effects and the biological effects of the radiation.

FOREWORD

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

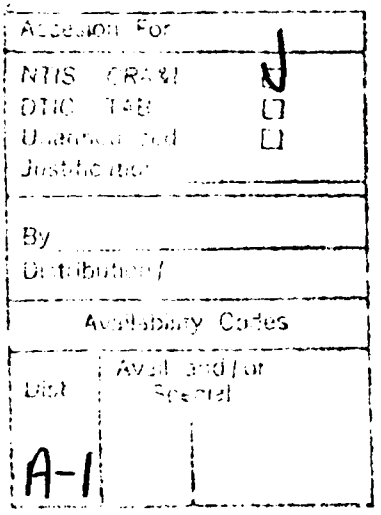


TABLE OF CONTENTS

The biological effects of ionizing radiation are mediated via the generation of damaging oxidizing free-radicals in the cell. Achieving effective chemical protection against these radicals in normal tissues has long been a goal of many researchers. The most widely studied of these "Antiradiation Compounds" is the class of compounds known as aminothiols, especially those having the generalized structure: $RHN(CH_2)_nNH(CH_2)_xSH$. The most promising drugs for the treatment of humans are the thiol-blocked phosphate-derivatives of these aminothiols, known as "aminophosphorothioates". The most widely studied of these compounds is WR-2721, the active form of which is the free aminothiol, WR-1065 (1). The application of such drugs to humans has so far been limited by their toxicity. The U.S. Army sponsored a program in which over 4000 compounds were synthesized and tested in mice (2,3). Unfortunately, the effective exploitation of the vast resource of potentially useful antiradiation drugs that have already been synthesized is greatly hampered by the lack of clear understanding of their mechanisms of action. Scavenging of free-radicals, H-atom donation, induction of hypoxia, and enhancement of cellular recovery/repair processes have all been proposed (4,5) to contribute to radioprotection.

The purpose of these studies has been to try and unravel some of these underlying processes that result in radioprotection. The work is discussed in 2 sections - studies with cultured mammalian cells treated in vitro, and studies with animal tissues treated in vivo. The fact that we will be examining both model in vitro and actual mouse tissue systems using the same basic analytical approach and radioprotective compounds allows a critical appraisal of the data. This approach will also enable us to address the validity of extrapolating effects determined on the basis of cell culture studies with such drugs to the in vivo situation, especially in view of the current debate as to the cellular nature of damage to normal tissues. The in vivo studies allow us to examine the effects of these drugs on radiation damage to tissues at three levels: molecular damage, sterilization of clonogenic cells, and functional response of the tissue or animal. Since there is an extensive literature suggesting that genomic DNA is the critical target for ionizing radiation (6,7), a significant amount of our time has been spent examining the effect of the compounds on various types of DNA damage and on the correlation of these effects with cell survival data.

A. STUDIES WITH CULTURED MAMMALIAN CELLS

First, I will review our progress so far with cultured Chinese hamster ovary (CHO) cells *in vitro*. As outlined in the previous report (8), the CHO cell data have provided important insight into the mechanism of radioprotection and have also contributed greatly to our ability to evaluate the *in vivo* data. We therefore set out to accumulate more detailed structure-activity relationships for a wider range of compounds. The results for the first compound that we characterized, WR-1065, have now been published (9). Similarly, the data for its terminal N-methyl derivative, WR-255591, have now been completed and accepted for publication (10). Since these 2 drugs were extensively discussed in our previous Annual Report (8), they will be used more for comparative purposes in this present report. We have now characterized several additional compounds, including the thiol dithiothreitol (DTT) and the aminothiols WR-151326 (the dephosphorylated derivative of WR-151327) and cysteamine for their ability to protect CHO cells from the induction of radiation-induced DNA single-strand breaks (SSBs) and double-strand breaks (DSBs). As in each of our previous studies we have compared the resulting protection factors (PFs) for these lesions with the PFs for cell killing. The structures of the drugs studied thus far are shown in Table 1.

A1. Cell Survival

The ability of WR-255591 to protect CHO cells irradiated on ice is shown in Fig. 1. Cells pretreated with 6 mM WR-255591 at 37°C for 30 min were protected by a factor of 2.35. When the cells were irradiated in drug-free media on ice and then immediately treated with 6 mM WR-255591 for 60 min at 37°C, there was no effect on survival (Fig. 1). When the cells were irradiated at 37°C, the 30-min pretreatment with WR-255591 protected with a PF of 2.43, while cells receiving both a pre- and posttreatment with the drug were protected by a factor of 2.34 (Fig. 2). The PF was therefore approximately 2.4 in all protocols where cells were pretreated with drug, regardless of the temperature at irradiation, whereas the presence of the drug after irradiation had no effect on survival. Each of these effects was also true for WR-1065 (9). The ability of WR-151326 to protect CHO cells is shown in Fig. 3. Like WR-255591, WR-151326 is methylated at the N-terminus but has an additional methylene group at the SH end of the molecule (Table 1). Pretreating with 6 mM WR-151326 for 0.5 h resulted in a 2.75-fold protection. As for the other 2 drugs, the degree of protection by WR-151326 did not depend on the temperature of the cells at the time of irradiation.

When the survival curves for WR-1065 were analyzed in detail prior to their publication (9) we chose to use two models--the simple multitarget (MT) model: $S(D) = 1-(1-e^{-D/D_o})^n$ and the linear-quadratic (LQ) model: $S(D) = e^{-\alpha D - \beta D^2}$, where S(D) is the surviving fraction as a function of dose D. The parameters of the MT model, D_o, D_q, and n, were determined by linear

regression analysis of the exponential portion of the survival curve. D_o (the dose required to reduce S by a factor of e^{-1}) was determined as the inverse of the slope and n (the extrapolation number) was determined by extrapolation of the exponential region to the y-axis. D_q was calculated as $D_o \ln(n)$. Values of α and β, the LQ parameters, were determined by replotting the survival data as $-\ln(S)/D$ versus D. This yielded a line of slope β and y-intercept α. PFs for cell killing were calculated as the D_o or α/β value obtained after treatment with radiation plus WR-1065 divided by the value after treatment with radiation alone. The values for each of these parameters for WR-1065 (4 mM) are shown in Table 2. Cells irradiated after pretreatment with the radioprotector were protected with a PF of 2.1, while cells receiving both a pre- and post-treatment were protected by a factor of 2.0 as determined from the ratio of D_o values from the MT model. PFs based on the α/β values for cells receiving pretreatment alone or pre- and post-treatment were 1.8 and 2.1 respectively (Table 2). PFs of between 1.8 and 2.1 were obtained irrespective of the model--MT or LQ--used to analyze the survival curves. Pretreatment with the drug caused an increase by a factor of 2 for both $1/\alpha$ and $1/\beta^{0.5}$ suggesting that protection is dose-modifying over this dose range. A similar analysis of the data for WR-255591 has now been made, and again the drug appears to be essentially dose-modifying when analyzed by either the LQ or MT model. The data for the other drugs have not, as yet, been analyzed in such detail.

The effect of increasing concentrations of four free-thiol drugs and of the phosphorothioate derivative of WR-255591 (WR-3689) on the survival of CHO cells is shown in Fig. 4. The cells were irradiated with 10 Gy at 37°C immediately following a 30-min pretreatment with the drug. There is a clear gradation of the free-thiol drugs: compounds with no charge, as illustrated by DTT, require relatively high concentrations to achieve a given level of protection. The monocationic aminothiol cysteamine protected at somewhat lower concentrations, and the doubly-charged "WR" compounds all protected efficiently at relatively low concentrations. As discussed elsewhere (4,11) a consideration of kinetic evidence implies that there must be some mechanism whereby compounds such as WR-1065 are concentrated close to the DNA. However, as will become apparent, this effect appears to result in an alteration not only in the magnitude of protection but also in the mechanism by which the drugs protect cells at the molecular level.

It is interesting to compare the abilities of WR-255591 and of its phosphorothioate derivative WR-3689 to protect cultured CHO cells. The properties of WR-255591 and WR-3689 closely parallel those of WR-1065 and WR-2721. The free-thiol compound (WR-255591) efficiently protected the cells while the phosphorothioate (WR-3689) protected minimally even at a concentration as high as 10 mM (Fig. 4). Similarly, WR-2721 usually affords little protection to cultured cells, while its free-thiol WR-1065 is an effective protector (12). Livesey and

co-workers (13) have shown that activation of WR-3689 to the free thiol (presumably WR-255591) in vitro using alkaline phosphatase results in efficient radioprotection of a RIF-1 fibrosarcoma cell line; again, this parallels the situation reported for WR-2721 in V-79 cells (14). More recent detailed studies (1,15) strongly reinforce the idea that the reduced form of WR-1065 is the form taken up and responsible for radioprotection when V-79 cells are incubated with either WR-1065 or with WR-2721 plus alkaline phosphatase. The recent data of Livesey et al. (13) indicate that cleavage of the phosphate group of WR-3689 is again a prerequisite for the transport of the drug into the cell. It therefore seems to be a general finding that the phosphorothioate group must be metabolized to the free-thiol before the drug can function as a radioprotector.

Figure 5 shows the time course of radioprotection by each of the drugs. Protection by the simple thiol DTT and by the simplest aminothiol, cysteamine, was extremely rapid and essentially complete by 5 min of exposure. For each of the aminothiols with multiple N-atoms, however, longer times were required to achieve maximum protection, usually on the order of 15 minutes. The time dependency of radioprotection for WR-1065 (Fig. 5) would appear to be faster than that reported for WR-1065 by Purdie (12). Our studies (Fig. 5) also indicate that the time course of protection by WR-255591 and WR-1065 are similar, suggesting that the drugs have equivalent kinetics of uptake in these cells.

A2. Single-Strand Break Induction

In order to establish whether the modification of DNA SSBs could be quantitatively related to the effect on survival under the same conditions we used the sensitive alkaline elution methodology (16). In one series of experiments we treated CHO cells with various concentrations of each drug for 30-min at 37°C before irradiation at 37°C. Pretreatment with either DTT, WR-1065, or WR-255591, considerably reduced the yield of DNA SSBs. Figure 6 shows a correlation plot relating the effects of these three drugs on SSB induction and cell survival: in this figure the drug concentration is being increased from bottom left through upper right. For DTT there was a reasonable correlation between the modification of SSBs and survival. For both WR-1065 and WR-255591, however, this correlation was very poor; both drugs protected against SSB induction but to an extent that was significantly less than their effect on cytotoxicity under equivalent conditions. For 6 mM WR-151326, the PF for DNA SSBs (1.78 $\pm$ 0.10) measured for cells irradiated at 37°C (not shown) was again much smaller than the PF for cell killing (~2.75).

In a second series of experiments, the drugs were evaluated for their ability to protect against SSB induction in iced cells, a situation where no repair of SSBs could occur during irradiation. When cells were incubated with 6 mM WR-151326 for 30 min at 37°C and then chilled before being irradiated with 5 Gy of γ-rays, the resulting PF was 1.68 $\pm$ 0.15 (Fig. 7). The PF for

SSBs was, therefore, still much lower than that for survival (PF of 2.75), as indeed was the case for both WR-1065 and WR-255591 (Table 3). For cysteamine and DTT, on the other hand, the PFs for SSB induction were much closer to those for survival (Table 3 and reference 17). Typical data for these drugs are shown in Fig. 8, again, the cells being irradiated on ice with 5 Gy. Our original rationale for studying DTT and cysteamine was based on the fact that both of these drugs have been studied previously by other workers: not only were their results interesting per se but they allowed us to "calibrate" our own data with the newer compounds (such as WR-151326) against existing data. Held et al. (18) have shown that DTT appears to modify radiation cytotoxicity in a way that correlates with its effects on DNA SSB-induction as measured using alkaline elution, suggesting that this drug is operating as a classical dose-modifying compound. Our interest in this compound was further stimulated by the report of Smoluk et al. (19) who studied the binding of various radioprotectors to DNA and concluded that both WR-1065 and cysteamine would bind to DNA under physiological conditions; DTT, on the other hand, would not bind to DNA under such conditions. Our data for DTT (Table 3 and reference 17) agree closely with those of Held et al. (18) insofar as this drug does indeed appear to reduce the efficiency of SSB-induction in proportion to its effects on cell killing.

A3. Double-Strand Break Induction

We have also measured the effect of each drug on the yield of DNA DSBs. Using the pH 7.0 variation of the neutral elution methodology, the dose-responses with or without the drugs were always linear; PFs were therefore calculated from the ratio of slopes using linear regression analysis. A 30-min pretreatment with 4 mM WR-1065 protected against DSB induction by a factor of 1.6 (9), 6 mM WR-255591 (Fig. 9A) protected by a factor of 1.8 (10). Our preliminary data for and 6 mM WR-151326 indicate a protection factor of approx. 2.8 (Table 3). While the PFs for DSBs for all 3 aminothiols are therefore somewhat closer to those for survival measured under the same conditions (2.0, 2.3, and 2.75, respectively, Table 3) than were the PFs for SSBs, for both WR-1065 and WR-255591 they still underestimated the degree of modification of survival; however, in the case of WR-151326 there seems to be a reasonable correlation with DSB induction. Figure 9B shows the results for 6 mM WR-255591 when the pH 9.6 version of the neutral elution method was used to measure DNA DSBs. Using the pH 9.6 assay, the dose responses were always curvilinear (Fig. 9B); protection factors were therefore calculated as the ratio of doses required to produce iso-effect at several levels of effect. In this case, a 30-min pretreatment with 6 mM drug protected by a factor of 1.70 at each level of effect. A similar result for WR-1065 has recently been reported by Sigdestad et al. (20).

For cysteamine and DTT our prediction was that the PF for DSBs should be similar to that for both cell killing and DNA SSB induction. The pH 7.0 DSB-induction data for cysteamine are shown in Fig. 10. Although the data are not yet completed, the

preliminary numbers in Table 3 tend to support this prediction, again suggesting that these two thiols may be acting more as "dose-modifiers" of DNA damage. Again, this agrees with recent data for mouse L or V79 cells treated with cysteamine which used a pH 9.6 protocol (21,22), where there was a close correlation between protection against DSB induction and cell killing.

A4. Single-Strand Break Repair

We also examined the effects of each drug on DNA SSB rejoining. In all cases the cells were irradiated with 5 Gy. Both WR-1065 (9) and WR-255591 (10) retarded the rate of SSB rejoining when present at the time of irradiation: the results for 6 mM WR-255591 are shown in Fig. 11. However, rather than reflecting a "repair inhibition", this effect may well result from the pretreatment altering the types of lesions induced in the DNA, as discussed later.

Post-irradiation incubation with either WR-1065 or WR-255591 also resulted in slower SSB rejoining even though post-treatment with these drugs had no effect on cytotoxicity (reference 9 and Fig. 1). This strongly suggested to us that these post-irradiation effects may not represent a true inhibition of the rejoining of γ-ray-induced SSBs, but rather may be due to additional breaks introduced during the repair incubation. Such lesions may in fact be produced via H_2O_2 resulting from enhanced autoxidation of the drug in irradiated cells (23) since the inclusion of catalase and desferal greatly reduced these post-irradiation effects for both WR-1065 (9) and WR-255591 (Fig. 12). We have now examined in some detail the effect of various scavengers/antioxidants on the level of breaks induced by various thiols. These data are discussed in Section A6.

A5. Double-Strand Break Repair

To measure DNA DSB rejoining, the cells were irradiated on ice with 100 Gy. DSBs were rejoined at the same rate by cells irradiated and/or repairing in the presence of WR-1065 as they were by cells that had not been exposed to the drug (9). Recent studies with WR-1065 by Sigdestad et al. (20) indicated a similar lack of effect. Studies of the effect of WR-255591 on DSB rejoining (10) revealed a very similar picture, as shown in Fig. 13; although the drug treatment lowered the initial level of DSB induction it had no effect on the rate of their removal. Thus, it seems to be a more general observation that the aminothiols have no detectable effect on DSB rejoining and thus that an altered rate of DSB rejoining does not appear to contribute to radioprotection.

A6. Effect on PLD Recovery

It is generally believed that cell killing is not governed solely by the level of damage inflicted to the cell, and that subsequent repair/recovery processes can modify the biological effects of the radiation (24). Three effects of aminothiols could affect PLD recovery: (a) their inhibition of cell progression; (b) their production of a shift in the spectrum of

DNA lesions, and (c) their effect on pH. The first of these effects, perturbation of cell progression, which had been reported with cysteamine (25,26), may alter PLD recovery by allowing additional time for the repair of potentially lethal lesions that would normally undergo fixation or conversion to lethal types of damage during replication had the cells been able to progress normally. Several drugs have now been evaluated using flow cytometry for their effect on cell progression. We confirmed the report (27) that WR-1065 perturbed cell-cycle progression by arresting cells in S-phase and possibly also in G_2 (9). A prediction from our studies is that agents such as DTT and WR-168643, which do not (or should not) bind to DNA, should have no effect on progression at nontoxic concentrations. For WR-1065, which binds relatively strongly to DNA (19), cell progression is indeed perturbed. It will therefore be critical to test the generality of this effect for other compounds (i.e., multi-cations).

We have recently begun to use the hypertonic salt fixation technique to examine the effect of these drugs on PLD recovery. Our preliminary data with WR-1065 are shown in Fig. 14. However, it should be stressed that many aspects of these data require clarification before major conclusions can be drawn. The protocol involves treating with hypertonic salt solution (0.5 M; medium with NaCl added) for 20-min at 37°C immediately after irradiation, a procedure with "fixes" a sector of PLD in log-phase cells (28) and enhances their radiosensitivity. When cells were pretreated with WR-1065, irradiated and immediately treated with hypertonic salt, they were sensitized to a greater extent than were the control cells, with the result that the PF for survival was significantly reduced compared with cells that were not treated with hypertonic salt. In fact, the PF decreased from 2.1 for control cells to 1.6 for salt-treated cells. This latter value, remarkably, is the same as the PF for DSB induction (Table 3). While this may be purely coincidental, it is nonetheless tempting to speculate that this could reflect a two-stage mechanism for protection, first by reducing lesion induction followed by an enhanced recovery process by one of the mechanisms discussed above. However, for the simple thiol DTT, where the effect on DNA strand-break induction and cell survival correlated closely, we would therefore predict that the salt treatment should have no effect on the PF for cell survival.

A7. Strand-Breaks Resulting from the Drugs Alone

Our initial interest in the DNA-damaging effects of the aminothiols themselves arose from the observation that both WR-1065 and WR-255591 at concentrations greater than 4 mM appeared to produce breakage of the control (unirradiated) DNA. At the time this was regarded as an annoyance which had to be compensated for in the radiation studies, and it was not until later that we realized that this effect may be important to understand in its own right. This was also becoming more important when we realized that mathematical correction for drug-induced SSBs may not be appropriate since the rate of

autoxidation of the aminothiol may be enhanced by the radiation (23). We therefore spent some time characterizing these breaks. Our approach was to manipulate their induction through the use of various enzymes or scavengers of free-radical/oxidative intermediates. For example, catalase is an enzyme that destroys H_2O_2, while superoxide dismutase (SOD) inactivates superoxide, $O_2^{\cdot -}$. Desferal and orthophenathroline modulate oxidative stress by scavenging iron (Fe^{++}/Fe^{+++}) which is usually involved in the redox-cycling of activated oxygen species. These studies were performed in association with Dr. Orazio Cantoni of the University of Urbino, Italy. This is illustrated for several thiols in Figs. 15-17. In this case, orthophenanthroline was the agent used to modulate the DNA-damaging effect of the thiol. Figure 15 shows the time course of development of DNA breakage as a function of continuous exposure to either 10 mM WR-255591, 10 mM WR-1065 or 50 mM DTT. On the basis of these data, a 30-min pretreatment was selected for the investigation of the effect of various modulating agents. As shown in Fig. 16, co-treatment with as little as 10 μM orthophenanthroline virtually eliminated the induction of SSBs, suggesting that Fe^{++}/Fe^{+++} is indeed important in the oxidation of these thiols. A similar effect was observed for 100 mM cysteamine (Fig. 17) and with the other scavengers such as desferal and catalase.

A8. Role of glutathione in radioprotection

Levels of endogenous thiols, and particularly of glutathione (GSH), which is the primary non-protein thiol in mammalian cells, could be an important determinant of the degree of protection achieved by the addition of exogenous radioprotectors. We have used the specific GSH-depleting agent buthionine sulfoximine (BSO) to investigate the role of GSH in the radioprotection process. So far these studies have been restricted to CHO cells. In these experiments we depleted GSH to a known amount of its control value and then determined whether there was an associated alteration in the PF (for SSBs, DSBs or cell killing) obtained with the subsequent addition of WR-1065. We used a concentration of BSO of 0.5 mM, and the exogenous radioprotector (4 mM WR-1065) was given 24 h later when the GSH levels had been depleted to less than 0.1% of control; 0.5 h later the cells were irradiated with various doses of γ-rays and then assayed for cell survival. The survival curves (Fig. 18) indicate that the BSO-treated cells are slightly sensitized to the radiation. Although the addition of WR-1065 protects these glutathione-depleted cells, this was to a much lower degree than the control cells were protected. The PF for control cells was 2.0 while the PF for BSO-treated cells was only 1.6. These data suggest that GSH may indeed be required for WR-1065 to exert its full radioprotective effect. Whether this is due to an enhanced oxidation of the WR-1065 in GSH-depleted cells or to other effects, for example an impaired level of PLD recovery in GSH-depleted cells, is presently being investigated, as outlined in Section D3.

Thus far, the DNA damage studies on BSO-depleted cells are only preliminary. The data suggest that GSH itself is not a

very effective intracellular radioprotective agent since extensive depletion of GSH does not appear to result in a significant enhancement of SSB induction. The initial experiments indicate that the WR-1065 dose-dependence of protection against SSB-induction appears to be very similar in GSH-depleted and control cells.

A9. Role of Polyamines in Radioprotection

The polyamines are a potentially important factor in radioprotection with respect to both their effect on chromatin structure and their potential effect on the DNA-binding capability of the aminothiols (8). We investigated the effect of polyamine levels on the ability of WR-1065 to protect CHO cells, using the drug γ-difluoromethyl ornithine (DFMO) to deplete polyamine levels. The cells were exposed to the DFMO for 48 h (1 mM). At this time the polyamine levels were measured by HPLC; putrescine and spermidine were completely undetectable while spermine levels were virtually unchanged from their control value. The DFMO treatment slightly sensitized the cells to γ-rays (Fig. 19). When the WR-1065 was added to the DFMO-treated cells they were protected by a factor of 2.4 (Fig. 19). Thus, the WR-1065 appears to be able to offset the sensitizing effect of the DFMO, virtually restoring the control survival curve. Although the PF increased from 2.0 for control cells to 2.4 for DFMO-treated cells, the depletion of polyamines did not result in an "overprotection" that we might have expected if the polyamines and aminothiols were in competition with each other. This may well reflect the fact that spermine levels were not depleted by the DFMO treatment. Experiments are in progress to examine the effect of spermine depletion on radioprotection.

A10. Neutrons

The ability of aminiothiols to protect against injury from high-LET radiations has been much less frequently studied than γ-rays, although such investigations may provide important insight into the mechanism of radioprotective action in addition to their obvious intrinsic value. So far, our studies have been restricted to an examination of the effects of WR-255591 on cell killing and on DNA damage induction after irradiation of CHO cells with cyclotron-generated 42-MeV fast neutrons. Pretreatment of cells with 6 mM WR-255591 for 0.5 h resulted in a 1.37-fold protection against the lethal effects of neutrons (Fig. 20). The data obtained for γ-rays under identical conditions are shown for comparison; in this case the PF for survival was 2.3. Under these same conditions, pretreating with WR-255591 protected against DNA DSB induction by neutrons by a factor of 1.38 (Fig. 21) using the pH 7.0 neutral elution method and against DNA SSB induction by a factor of 1.37 (Fig. 22).

On the basis of the postulated mechanism(s) of radioprotection, we would have expected that protection should decrease for higher LET radiations, as indeed it did. For example, if induction of anoxia is important, neutrons show a much lower OER than γ-rays (the OER is 1.4 for the present

neutron beam and cell line; unpublished data). Similarly, if enhanced PLD recovery is important (Section A6), the drugs would be expected to show a reduced ability to radioprotect against neutron damage on the basis that PLD recovery should decrease with increasing LET. Again, if scavenging of $OH^{\cdot}$ radicals is important, neutrons impart much less of their biological effect via indirect action so they should be less easy to modify by thiols. Finally, if oxygen fixation versus H-atom donation (repair) is important, then the PF should again be reduced in parallel with the OER; in the present experiments the PF for 6 mM drug was in fact equal to the OER, so it will be interesting to see what happens at higher drug concentrations. Exactly why these events should result in the essentially dose-modifying behavior for neutrons, i.e., PF (survival) $\simeq$ PF (SSB) $\simeq$ PF (DSB), whereas for γ-rays PF (survival) $>$ PF (DSB) $>$ PF (SSB), is unclear at present.

A11. Conclusions

As we accumulate more and more data we find that the unmistakeable conclusion from these studies is that protection of aerated CHO cells from γ-radiation by aminothiols such as WR-1065, WR-255591, or WR-151326 is not accompanied by a uniform lowering of the initial levels of all types of γ-ray-induced DNA lesions. The PFs for either SSBs or DSBs are always lower the PF for survival (with the possible exception of WR-151326 and DSBs), suggesting either that some particular subset of lesions which are crucial for cell killing may be selectively protected by the agent or that these drugs may stimulate cell recovery processes. The number of drugs that have been studied are beginning to enable us to draw some general conclusions. The simpler thiols such as DTT which bear no formal charge and the simpler aminothiols such as cysteamine appear to lower the levels of DNA lesions in proportion to their effect on cell survival. On the other hand, the effects of protective compounds such as WR-255591 and WR-1065 are numerous, and their protective effect is probably the result of a complex interaction between several of these effects. These data have led us to believe that the complex dicationic aminothiols such as WR-1065 and WR-255591 may exert an important part of their effect on cellular radiosensitivity by binding to DNA. Thiols such as DTT, on the other hand, which lack the amino function and thus the ability to bind to DNA, probably protect entirely by scavenging damaging free-radicals and by proton donation to DNA radicals.

B. STUDIES WITH MOUSE JEJUNUM

Next I will review our recent progress on the mechanism of radioprotection of mouse jejunum. Our first study characterized this tissue for its response to γ-radiation in the absence of radioprotective drugs (29). Our earlier studies of SSBs in mouse jejunum (30) had used an adaptation of the alkaline elution methodology that characterized the response of the entire cell populations that comprised the tissue. However, the biological effects of radiation probably depend on the response of specific target cells within the tissue and which, in the case of the jejunum, are most likely to be the jejunal stem cells that maintain and/or repopulate the tissue after injury. These target cells may well have their own discrete DNA damage and repair characteristics. To examine subpopulations of cells within the jejunum we developed a method in which we pulse-label those cells that are rapidly proliferating by injecting the mouse with tritiated thymidine (^{3}H-TdR). By varying the time between injection of the radiolabel and irradiation, SSBs can be measured in different cell populations of interest. The response of these subpopulations should give a better indication of the biological response of the tissue than should the response of the entire tissue, and this assay was used for the DNA damage experiments in the studies described below.

B1. Jejunal Microcolony Assay

So far we have characterized two phosphorothioates, WR-2721 (31) and WR-3689 (32), and their respective free-thiol metabolites, WR-1065 and WR-255591, for their ability to protect mouse jejunal cells in vivo. We used the jejunal microcolony assay to measure the effect of the drugs on the survival of clonogenic crypt cells (33). The results obtained for each drug tested thus far using the jejunal microcolony assay are collected in Table 4. The results with WR-2721 were described extensively in our previous report (8). However, its N-methyl derivative, WR-3689, has been relatively poorly characterized in this assay so we undertook a detailed examination of both the drug-dose dependence and also the time-course of its protection of mouse jejunal crypt cells. WR-3689 is a promising radioprotective drug insofar as it protects normal tissues effectively at relatively non-toxic doses, as determined either by its ability to protect mice from a dose of whole-body irradiation (WBI) sufficient to produce 100% mortality in 30 days (3) or by its ability to protect the hematopoietic system using an $LD_{50/30}$ assay (34). It was also reported that WR-3689 was as effective as WR-2721 for protecting bone marrow but not jejunum, using an $LD_{50/30}$ and $LD_{50/7}$ assay, respectively (35). Preliminary studies indicate that WR-3689 is somewhat less toxic than WR-2721 (3,35) while the therapeutic index may be at least as good based on their relative effect on hematopoietic damage and on protecting a murine mammary carcinoma growing in the leg (34).

First, we examined the effects of different doses of WR-3689 on the number of surviving crypt cells after a single dose (14 or 18 Gy) of radiation. Based on recent studies of the tissue

pharmacology of WR-3689 (36), a constant time of 30 min before irradiation was used. The results are shown in Fig. 23; at both radiation doses there was a dramatic protection of the crypt cells for doses of WR-3689 up to 600 mg/kg, at which point the curves appeared to be reaching a plateau.

Next, we examined the effect of varying the timing of treatment with the drug relative to the irradiation. These data are shown in Fig. 24; in each case the drug was given ip at a dose of 200 mg/kg at different times prior to WBI with 14 Gy of γ-rays. Protection was achieved fairly rapidly, with maximum protection being achieved when the drug was administered 30 min prior to irradiation. The degree of protection declined again when the drug and radiation were separated by longer intervals, and by 2 h the level of crypt-cell survival was only slightly greater than control (no drug) levels. We also selected certain treatment protocols to generate complete radiation dose-response curves for crypt cell inactivation. Two doses of WR-3689 were used -- 400 mg/kg and 800 mg/kg. The results were shown in our previous Annual Report (8); briefly, WR-3689 (400 mg/kg) administered 30 min before WBI gave a PF of 2.0, while a dose of 800 mg/kg protected with a PF of 2.5, based on the ratios of the D_o values (Table 4).

We have shown here (Figs. 23 and 24) that WR-3689 is extremely effective in protecting mouse jejunal crypt cells irradiated in situ when the endpoint of clonogenic crypt cell survival is considered; indeed, the data suggest that WR-3689 was at least as good a protector as WR-2721 at an equivalent administered dose. For example, at a dose of 400 mg/kg the PFs for WR-2721 and WR-3689 were 1.8 and 2.0, respectively (Table 4). As shown by Rasey and coworkers (36), the level of drug achieved in the small intestine 30 min after ip injection is almost twice as high for WR-3689 compared with WR-2721 when expressed as a percentage of the injected dose per gram of tissue. Thus, the effectiveness of these drugs in a clonogenic assay appears to parallel their biodistribution; however, Rasey et al. (35) have determined WR-3689 to be less effective than WR-2721 in protecting against intestinal radiation syndrome. In view of this apparent anomaly, we feel that it will be important to establish animal survival data in our own mouse strain and using the same conditions and batch of WR-3689. These experiments are in progress.

The major advantage of WR-3689 over WR-2721 appears to be its lower toxicity, especially since protection could be increased by further increasing the drug dosage (Fig. 23) beyond the levels that can be tolerated for WR-2721. It is also apparent that, while the protection appeared to be leveling out at doses of drug in excess of 600 mg/kg (Fig. 23), this was in contrast to the situation with WR-2721 where crypt-cell survival was virtually independent of drug dosage above 300 mg/kg (37). The two drugs also differed in the dependency of protection on the time separation between drug injection and irradiation. For

WR-2721 (37) protection was near maximal within 5 min after drug injection and remained high for up to 1 h, after which it decreased relatively slowly, the crypt levels returning to control value only when the drug was given approx. 6 h before WBI. For WR-3689, on the other hand, a distinct maximum crypt-cell survival was observed when the drug was administered 30 min before irradiation, with protection decreasing fairly rapidly at longer time intervals (Fig. 24), presumably as a result of the more rapid elimination of the active radioprotective metabolite compared to WR-2721. There was no evidence for the extended plateau of response that was observed with WR-2721 (37). There are several possible explanations for these effects, most notably differences in either pharmacokinetics, drug activation, or cellular uptake. Rasey and coworkers (36) have recently measured the pharmocokinetics of ^{35}S-radiolabeled WR-3689 in a variety of C_3H mouse tissues; maximal levels of the drug in the small intestine were indeed observed after 30 min and then declined slightly (but significantly) by 60 min. For WR-2721, these same authors (38) have shown that maximum accumulation in the gut was also at 30 min after injection, but in this case the level did not significantly decline from its peak value even after 2 h. At least with respect to the disappearance of protection, therefore, the distribution of ^{35}S appears to reasonably predict crypt-cell response. It should be remembered, however, that such data do not allow a selective analysis of the tissue levels of the free-thiols (WR-255591 and WR-1065) which should be a better predictor of their radioprotective behavior. We know that in cultured CHO cells the time course of the development of radioprotection appears to be similar for WR-255591 and WR-1065 (Fig. 5), so this effect may not have an intrinsic cellular basis. Furthermore, the relative level of WR-3689 in the small intestine after 30 min was almost twice as great as that measured for WR-2721 (36). It therefore appears that while the relative levels of the drug achieved in <u>different</u> tissues does not usually predict well for the protection of that tissue, perhaps as a result of differences in their oxygenation (39), measurements of the relative levels of structurally similar aminophosphorothioates in a <u>single</u> tissue may provide a reasonable estimate of their protective capability.

B2. <u>Initial Levels of SSB Induction</u>

Three drugs have now been tested for their ability to protect against DNA SSB induction in the jejunum (Table 4). The data for WR-2721 and WR-1065 have now been published (31) and will only be briefly summarized here. Both WR-2721 and WR-1065 (400 mg/kg) when given 15 or 30 min prior to WBI reduced the amount of damage induced both in the whole jejunum and the jejunal crypts (Table 4). However, in each case the effect was relatively small (PFs of less than 1.2 in all cases) compared to the associated effect on crypt-cell survival. SSB levels have also been measured in animals treated with WR-3689 (8,32). WR-3689 (400 or 800 mg/kg) given 30 min prior to irradiation had virtually no effect on the number of SSBs induced either in the

whole jejunum or in the crypt cells despite the fact that it protected in the microcolony assay with a PF of 2.0 and 2.5 at these same doses (Table 4). It seems highly improbable to us that the radiosensitive target cells, which must be very adjacent to the proliferative zone, would show any dramatic difference from these proliferating cells. It therefore appears that the protection against jejunal crypt cell inactivation is not due to a corresponding reduction in the initial number of SSBs.

B3. Effect on SSB Rejoining

We also examined the effect of these drugs on the rate of rejoining of DNA SSBs. Both WR-1065 and WR-2721 (31), or WR-3689 (32) appeared to retard the rate of SSB-rejoining both in the whole epithelium and in the proliferating crypt cells. As discussed in Section A11, this may be a reflection of the drugs ability to alter the spectrum (and thus the repairability) of lesions induced, although it may also result from our present inability to regulate drug oxidation in vivo in such a way as we have been able to in vitro (Section A7).

B4. Conclusions

The studies with mouse jejunum remain something of an enigma. It is apparent that radioprotection of the jejunum has a cellular basis insofar as the modification of crypt-cell survival reasonably predicts the response of the animal in an $LD_{50/7}$ assay (40,41). However, the nature of the effects at the DNA (molecular) level responsible for this protection are apparently complex. It is equally apparent that for both WR-2721, WR-1065 (31) and WR-3689 (32) at all doses of drug or radiation studied, the PFs for initial damage either in the whole epithelium or in the proliferating crypt cells were much smaller than the corresponding PFs for survival of clonogenic cells obtained with the microcolony assay. There are several possible explanations for this. That the data simply reflect a lack of a relationship between SSBs and cell killing is not unreasonable since DSBs are probably more important in cell inactivation (42). It is nonetheless surprising that the reduction of these lesions did not parallel the modification of such "lethal" events. However, our studies using CHO cells (Section A) suggest that the aminothiols may selectively modify the induction of sub-sets of lesions, and the present data may simply be an unusually extreme extension of this observation whereby SSBs are not modified at all under these conditions.

A second possibility is that the low oxygen tension in the tissues may have an important influence on the relationship between SSBs and survival. Travis (39) has reviewed the literature suggesting that tissue oxygenation is an important factor in radioprotection in vivo. The above data may therefore reflect the poor oxygenation of mouse jejunal cells. Perhaps as a result of their radiobiological hypoxia, the efficiency of radiation-induced strand breakage in jejunal cells is already greatly reduced relative to that in aerated cell suspensions (43,44), so they may be relatively insensitive to further

WR-2721 (37) protection was near maximal within 5 min after drug injection and remained high for up to 1 h, after which it decreased relatively slowly, the crypt levels returning to control value only when the drug was given approx. 6 h before WBI. For WR-3689, on the other hand, a distinct maximum crypt-cell survival was observed when the drug was administered 30 min before irradiation, with protection decreasing fairly rapidly at longer time intervals (Fig. 24), presumably as a result of the more rapid elimination of the active radioprotective metabolite compared to WR-2721. There was no evidence for the extended plateau of response that was observed with WR-2721 (37). There are several possible explanations for these effects, most notably differences in either pharmacokinetics, drug activation, or cellular uptake. Rasey and coworkers (36) have recently measured the pharmocokinetics of ^{35}S-radiolabeled WR-3689 in a variety of C_3H mouse tissues; maximal levels of the drug in the small intestine were indeed observed after 30 min and then declined slightly (but significantly) by 60 min. For WR-2721, these same authors (38) have shown that maximum accumulation in the gut was also at 30 min after injection, but in this case the level did not significantly decline from its peak value even after 2 h. At least with respect to the disappearance of protection, therefore, the distribution of ^{35}S appears to reasonably predict crypt-cell response. It should be remembered, however, that such data do not allow a selective analysis of the tissue levels of the free-thiols (WR-255591 and WR-1065) which should be a better predictor of their radioprotective behavior. We know that in cultured CHO cells the time course of the development of radioprotection appears to be similar for WR-255591 and WR-1065 (Fig. 5), so this effect may not have an intrinsic cellular basis. Furthermore, the relative level of WR-3689 in the small intestine after 30 min was almost twice as great as that measured for WR-2721 (36). It therefore appears that while the relative levels of the drug achieved in <u>different</u> tissues does not usually predict well for the protection of that tissue, perhaps as a result of differences in their oxygenation (39), measurements of the relative levels of structurally similar aminophosphorothioates in a <u>single</u> tissue may provide a reasonable estimate of their protective capability.

B2. <u>Initial Levels of SSB Induction</u>

Three drugs have now been tested for their ability to protect against DNA SSB induction in the jejunum (Table 4). The data for WR-2721 and WR-1065 have now been published (31) and will only be briefly summarized here. Both WR-2721 and WR-1065 (400 mg/kg) when given 15 or 30 min prior to WBI reduced the amount of damage induced both in the whole jejunum and the jejunal crypts (Table 4). However, in each case the effect was relatively small (PFs of less than 1.2 in all cases) compared to the associated effect on crypt-cell survival. SSB levels have also been measured in animals treated with WR-3689 (8,32). WR-3689 (400 or 800 mg/kg) given 30 min prior to irradiation had virtually no effect on the number of SSBs induced either in the

whole jejunum or in the crypt cells despite the fact that it protected in the microcolony assay with a PF of 2.0 and 2.5 at these same doses (Table 4). It seems highly improbable to us that the radiosensitive target cells, which must be very adjacent to the proliferative zone, would show any dramatic difference from these proliferating cells. It therefore appears that the protection against jejunal crypt cell inactivation is not due to a corresponding reduction in the initial number of SSBs.

B3. Effect on SSB Rejoining

We also examined the effect of these drugs on the rate of rejoining of DNA SSBs. Both WR-1065 and WR-2721 (31), or WR-3689 (32) appeared to retard the rate of SSB-rejoining both in the whole epithelium and in the proliferating crypt cells. As discussed in Section A11, this may be a reflection of the drugs ability to alter the spectrum (and thus the repairability) of lesions induced, although it may also result from our present inability to regulate drug oxidation in vivo in such a way as we have been able to in vitro (Section A7).

B4. Conclusions

The studies with mouse jejunum remain something of an enigma. It is apparent that radioprotection of the jejunum has a cellular basis insofar as the modification of crypt-cell survival reasonably predicts the response of the animal in an $LD_{50/7}$ assay (40,41). However, the nature of the effects at the DNA (molecular) level responsible for this protection are apparently complex. It is equally apparent that for both WR-2721, WR-1065 (31) and WR-3689 (32) at all doses of drug or radiation studied, the PFs for initial damage either in the whole epithelium or in the proliferating crypt cells were much smaller than the corresponding PFs for survival of clonogenic cells obtained with the microcolony assay. There are several possible explanations for this. That the data simply reflect a lack of a relationship between SSBs and cell killing is not unreasonable since DSBs are probably more important in cell inactivation (42). It is nonetheless surprising that the reduction of these lesions did not parallel the modification of such "lethal" events. However, our studies using CHO cells (Section A) suggest that the aminothiols may selectively modify the induction of sub-sets of lesions, and the present data may simply be an unusually extreme extension of this observation whereby SSBs are not modified at all under these conditions.

A second possibility is that the low oxygen tension in the tissues may have an important influence on the relationship between SSBs and survival. Travis (39) has reviewed the literature suggesting that tissue oxygenation is an important factor in radioprotection in vivo. The above data may therefore reflect the poor oxygenation of mouse jejunal cells. Perhaps as a result of their radiobiological hypoxia, the efficiency of radiation-induced strand breakage in jejunal cells is already greatly reduced relative to that in aerated cell suspensions (43,44), so they may be relatively insensitive to further

modification by radioprotective drugs. Alternatively the K-curves for SSB-induction and cell killing may not be coincident at lower oxygen tensions, and the survival characteristics may be changing more rapidly than SSB levels as a function of oxygen tension.

In summary, our more recent studies confirm that radioprotection of clonogenic jejunal crypt cells does not appear to correlate with the modification of DNA SSBs for a variety of aminothiols. Since it is highly unlikely that the stem cells are a unique population in terms of protection against DNA damage, this may well reflect that the drug is not resulting in a dose-modification of the DNA lesions. Tissue oxygenation may be the most important factor determining these relationships. For this reason, we examined the relationship between cell survival and DNA damage in the mouse bone marrow, a tissue that we have previously shown (43) to be relatively well oxygenated (radiobiologically). These data are discussed in the following section.

C. STUDIES WITH MOUSE BONE MARROW

We have now begun to examine these same processes, described in Section B for jejunal cells, in the mouse femoral bone marrow cells. The rationale for these experiments derives from the observation that this tissue appears to be, radiobiologically speaking, much better oxygenated than the jejunum and may therefore provide an *in vivo* system intermediate between the jejunal crypts and the CHO cells.

C1. Spleen Colony Assay

We have used the endogenous spleen-colony (E-CFU) assay devised by Till and McCulloch (45) to examine the ability of various drugs to protect bone marrow stem cells irradiated *in situ*. The data for WR-2721 are shown in Fig. 25. It is clear that the drug afforded considerable protection in this assay; however, the actual PF value depended strongly on the method of calculation and it will require further data to clarify this. WR-2721 (400 mg/kg i.p. given 30-min before WBI) protected in the E-CFU assay by a factor of 1.45 when a level of isoeffect of 5 E-CFU per spleen was chosen. However, the PFs based on the ratio of the D_o values appears to be somewhat greater than 2. Dr. Elizabeth Travis in our department has simultaneously performed the exogenous bone marrow assay and found that WR-2721 (200 mg/kg) given 30-min before WBI protected by a factor of 2.3. Under these same conditions she has found that the animals were protected in the $LD_{50/30}$ assay by a factor of 1.8.

C2. Initial Levels of SSB Induction

As for the jejunum, our first goal was to examine the possibility of using ^{3}H-TdR pulse-labeling to measure DNA SSBs in the proliferating bone-marrow cells. This was indeed possible, and the results were published along with those for jejunal cells (29). As with the jejunal crypt cells, those cells that were in a state of rapid proliferation rejoined their DNA SSBs more rapidly than the bulk differentiated cells. Next, we used this modification (29) of the alkaline elution assay to measure DNA SSB induction in mouse bone marrow cells irradiated *in situ*. The mice were injected with ^{3}H-TdR (1.0 μCi/g) 6 h prior to irradiation. The results are shown in Fig. 26. In marked contrast to the data with jejunum, WR-2721 (400 mg/kg, i.p., 30-min pre-irradiation) protected markedly against SSB induction both in the proliferating cells and in the whole cell population. The PFs for DNA SSB induction were generally on the order of 1.55 independent of the assay used. Thus, the PFs in the different cell populations appeared to be similar.

C3. Conclusions

The data with mouse bone marrow are still incomplete and therefore no quantitative conclusions can be made at this point. Qualitatively, however, the data strongly support our predictions from Section B4 that bone marrow cells may well be better oxygenated than jejunal cells and that this in turn modifies the relationship(s) between cell killing and DNA damage (and the effects of aminothiols thereon). These data also reaffirm our

belief that understanding exactly how oxygen modifies these effects should be a major objective of our future studies (Section D2).

D. SUMMARY, FUTURE PLANS AND SPECIFIC RECOMMENDATIONS

D1. Extension of structure-activity relationships through the use of additional model radioprotective compounds in vitro and in vivo.

A major objective in the coming year will be to extend our knowledge of the structure-activity relationships relating radioprotective ability and the ability to alter the induction and repair of particular types of DNA lesions. This will involve the selection of additional for study based on the structure-activity relationships accumulated this far. One of the criteria that we will use for selecting additional compounds is their predicted DNA-binding capability, which is most-likely related to (a) the number of amino-groups, (b) the distance between the amino groups, and (c) the degree of substitution of the amino groups. Compounds with variations in these features will be selected from the list published by Sweeney (2) for further investigation. The rationale for the selection of compounds is illustrated by the following case. We have for some time been studying the effects of the thiol DTT on cultured CHO cells (Section A). This compound has provided an excellent probe for understanding the ability of WR-1065/WR-255591 to modify the spectrum of DNA lesions induced by γ-rays. We had predicted that, for DTT, the effect of the drug should be dose-modifying, i.e., that the drug should affect survival, DSB and SSB induction equally. It is clear from Section A that this prediction has been reasonably well substantiated. Another potentially interesting compound is the bis sodium sulfinate trisulfide compound WR-168643 [$HO_2S(CH_2)_4SSS(CH_2)_4SO_2H$] which has an intriguingly high ability to protect animals from radiation lethality (3,46) Like DTT, WR-168643 has no nitrogen atoms and is uncharged at neutral pH, and therefore should not bind to DNA (19); in view of our proposed model, this type of compound should be of considerable importance mechanistically, especially since it can be ultimately used in the animal tissue studies. A second drug that we propose to investigate in the near future would be the free thiol of WR-44923 if such a compound is now available (since it dose not appear in reference 2). Alternatively, we could use a combination of WR-44923 and alkaline phosphatase. Our interest in this compound (and its phosphorothioate, WR-44923) stems from our observation (Table 3) that WR-151326, in which the SH and NH groups are separated by a 3-carbon chain, had unusual and interesting effects on SSB versus DSB induction. WR-44923 also has the 3-carbon chain but has the free terminal $-NH_2$ group rather than the N-methyl group and should therefore be an extremely interesting compound. We hope that through such an approach we may begin to understand the complex mechanisms by which the aminothiols exert their protective effects.

D2. Role of Oxygen in Radioprotection by Aminothiols

As discussed in Sections B4 and C, intracellular oxygen concentration is likely to be a major factor not only in radioprotection but also in determining the interrelationships between the modification of DNA damage and cell killing, so the

examination of the effects of radioprotectors on aerated cultured cells may not appropriately mimic the in vivo situation. We are currently using CHO cells as a model system to examine the effects of equilibration with various levels of oxygen on the subsequent effects of WR-1065 on γ-ray-induced cell killing and DNA damage. This approach is taking advantage of techniques that we have been developing in our laboratory whereby the cells can be equilibrated with O_2/N_2 mixtures of known composition and then treated sequentially with drugs and γ-rays without perturbing the equilibrium conditions.

Our own studies (Sections B and C) of the effect of WR-2721 and WR-3689 on mouse jejunal crypt and bone marrow cells (see Table 4) strongly reinforce the importance of understanding the role of oxygenation in radioprotection in vivo. We feel that such studies are best achieved through the use of model in vitro systems where the drug and oxygen concentrations can be carefully regulated. We already have significant background data showing the oxygen effect on cell survival, SSB and DNA-protein crosslink (DPC) induction (Fig. 27). These data were obtained using the apparatus shown in Fig. 28, which allows equilibration of both the cells and drug independently with the gas phase and then allows them to be mixed prior to irradiation. We are presently constructing similar K-curves relating each of the biological endpoints--cell survival, SSB and DPC induction--to the concentration of oxygen in the gas phase, using WR-1065 as the initial test drug. Thus, not only will the effect of O_2 concentration on the PF for cell survival be determined, but also we will be able to examine how this modification of survival correlates with changes in DNA damage. Only by such an approach can this important information be obtained and used to explain the (presently) anomalous data for animal tissues.

D3. Role of glutathione and polyamines in radioprotection.

Our preliminary data using BSO and DFMO to deplete glutathione and polyamines, respectively, were described in sections A8 and A9. These studies are proving to be extremely interesting and will be pursued during the coming year. The DNA damage aspects of these studies, which were outlined in the previous report (8), are currently ongoing, and should help to explain the current findings in the survival assay.

D4. Determination of the intracellular forms of the drugs that are responsible for these various effects.

All of our previous studies have involved measuring final endpoints such as cell survival or DNA lesions. Exactly what form(s) of the drugs correlate with these effects has not yet been determined. We propose to use an approach similar to that developed in Fahey's laboratory (e.g. see references 1 and 15) using HPLC technology to identify the intracellular forms of the various drugs.

D5. Investigation of the effects of different thiols in combination.

One aspect of radioprotection that was outlined in our original proposal is now in a position to be evaluated, namely the effect of combining different thiols on cell survival and DNA damage. In our preliminary studies we chose to use WR-1065 and DTT in view of their projected differences in mechanism. The cells are treated with 4 mM WR-1065 for 30 min. After this time the drug is washed off and the cells are treated for 5 min with various concentrations of DTT. By measuring the resulting survival we can determine whether the drugs are additive, synergistic, or even antagonistic, and thus gain further insight into their proposed mechanism(s) of action.

E. EXPERIMENTAL PROCEDURES

E1. Cell Culture Methods

CHO cells are maintained and treated in exponentially growing monolayer culture at 37°C in a humidified 5% CO_2 - 95% air atmosphere in McCoy's 5A medium (Hsu's modification) supplemented with 15% fetal bovine serum. For the DNA damage studies cells are labeled for 24-36 h with 2-^{14}C-TdR (0.01 μCi/ml; 50 mCi/mmol) followed by a 6-h incubation with label-free medium to chase the label into high molecular weight DNA.

E2. Cell Survival

Cell survival is determined using a clonogenic assay, surviving cells being assayed by their ability to produce colonies of 50 cells or more. For each experiment, the cells are plated in triplicate so that about 80-100 colonies are produced on each dish. All survival data are the average of between 3 and 6 separate experiments, and the standard error is displayed with the mean.

E3. Mice

C_3Hf/Kam mice are maintained in a specific-pathogen-free breeding colony. Mice of between 12 and 16 weeks of age and weighing approximately 30 g are used for all studies. For the DNA SSB measurments, the mice are injected i.p. with 1.0 μCi/g of ^{3}H-TdR 6 h prior to whole-body irradiation. Irradiation is always performed at the same time of day in order to minimize any possible circadian effects. The radioprotective agent is administered i.p. at various times relative to the irradiation. Animals are sacrificed by cervical dislocation at various times after irradiation, and the tissues (jejunum or bone marrow) are removed and immersed in ice-cold PBS containing 5 mM EDTA to inhibit any SSB repair. All cell suspension preparation procedures are conducted at ice-bath temperatures, and the tissues are maintained on ice in PBS containing 5 mM EDTA at all times after sacrifice, again to inhibit any repair of SSBs or degradation of the DNA. To prepare suspensions of jejunal epithelial cells, any undigested material in the lumen is first removed by flushing with cold PBS via a 15-gauge needle. The jejunum is then cut open along its length and the mucosal layer gently separated by scraping with a glass slide in a petri dish. The cells are suspended in PBS by gentle pipetting and filtered through cotton gauze. The resulting cell suspension is made up to 10 ml with ice-cold PBS, and then filtered through cotton gauze. Because of the difficulty in preparing true single-cell suspensions of jejunal epithelium without introducing mechanical damage to the DNA, these cell numbers can not be determined exactly; rather, between 0.6 and 1.0 ml of the suspension is used for alkaline elution analysis, the exact amount depending on the density of the suspension. Suspensions of femoral bone marrow cells are obtained by syringing the cells out of the tibias and femurs with PBS into a beaker, followed by passage through a 26-gauge needle. The cell suspensions are washed twice by sedimentation at 2000 rpm for 10 min in a refrigerated Beckman

TJ-6 centrifuge, resuspended in ice-cold PBS, and then filtered through cotton gauze. For bone marrow, the numbers of cells are counted with a hemacytometer.

E4. Alkaline Elution In Vitro

DNA SSBs in the cultured CHO cells are measured using alkaline elution (16). Approximately 8×10^5 ^{14}C-radiolabeled cells are layered onto 25-mm diameter, 2-um pore polycarbonate (PC) filters (Nuclepore Corp., Pleasanton, CA), and washed twice with ice-cold PBS containing EDTA (5 mM). The cells are then lysed with 10 ml of SDS lysis solution (0.025 M Na_4EDTA, 2% SDS, pH 9.7) containing proteinase K (0.5 mg/ml), and washed twice with 0.02 M EDTA solution, pH 10.3. The DNA is subsequently eluted in the dark with 0.1 M tetrapropylammonium hydroxide (TPAH) containing 0.02 M EDTA (free acid), pH 12.1, at a constant flow rate of 0.04 ml/min. Fractions are collected every 90 min for 15 h, resulting in a fraction volume of approximately 3.5 ml. Any DNA retained on the filter at the end of the elution time is recovered by heating for 1 h at 60°C in 1 M HCl (0.4 ml) followed by the addition of 0.4 M NaOH (2.5 ml) for 30 min. DNA remaining in the filter holder or barrel is recovered by flushing with 3 ml of NaOH solution. The amount of DNA in each fraction, as well as that remaining on the filter and that recovered from the interior of the filter holder, is assayed by liquid scintillation counting.

E5. Neutral Elution

DNA DSBs are measured using the conventional neutral elution method (47) which uses a pH of 9.6 or with a modification of that technique in which a pH of 7.0 is used for the eluting buffer and for all other solutions. The latter adaptation may reduce the contribution from various types of SSBs to the DSB measurements, while the former method enables a better comparison with existing data in the literature which have usually used of pH of 9.6 (e.g. 21,22).

E6. Alkaline Elution In Vivo

The alkaline elution technique (16) has been adapted for measuring DNA SSBs in cells from the mouse jejunal epithelium and bone marrow after irradiation of the tissues in vivo. These modifications (48,29) permit DNA SSBs to be determined in both the total and proliferating cell populations of the same sample of tissue. Approximately 5×10^6 cells are layered onto a 47-mm diameter, 0.8-um pore PC filter and lysed with a solution containing 2 M NaCl, 0.04 M Na_4EDTA, 0.2% Sarkosyl, and 0.5 mg/ml proteinase-K, final pH 10.0. After 0.5 h, the DNA is washed with 0.02 M EDTA solution and is then eluted in the dark with 0.1 M TPAH containing 0.02 M H_4EDTA, pH 12.1, at a constant flow rate of 0.04 ml/min. In some experiments the cells are lysed with 10 ml of sodium dodecyl sulfate (SDS) lysis solution (0.025 M Na_4EDTA/2% SDS, pH 9.7), rinsed twice 0.02 M EDTA, and the DNA is eluted with 0.1 M TPAH/0.02 M H_4EDTA/0.1% SDS, (pH 12.1). Any DNA retained on the filter at the end of the elution time is recovered by heating to 60°C for 60 min in pH 10.3 EDTA wash

solution. The DNA concentration in each of the 10 eluted fractions and that recovered from the filter is assayed either fluorometrically (using Hoechst 33258) or by liquid scintillation counting (48). Since the mice are labeled with ^{3}H-TdR 6 h prior to irradiation, the radiolabel is predominantly located in the proliferating cells of the jejunal crypts or bone marrow at the time of irradiation (29). The data obtained using the radioactivity assay therefore characterize DNA-damage in these cells only, while the data obtained using the fluorometric assay measure DNA SSBs in all of the cells in that sample of tissue. Data are the average of at least six separate experiments, and the standard error is displayed with the mean value.

E7. Calculation of Strand-Scission Factors

The relative number of strand breaks is determined by the equation: $SSF = -\log(f_x/f_o)$, where f_o and f_x are, respectively, the proportion of DNA retained on the filter for the unirradiated control and for the irradiated sample. For SSBs, an eluted volume of 21 ml is used for the calculation. It should be noted that, since the dose-response curves for SSB frequency versus dose are, in general, non-linear for CHO cells at low doses (e.g., 48), the repair kinetics, when expressed as a percentage of initial damage remaining, must be calculated directly from the calibration curve. This curvilinearity is also an important consideration in the determination of protection factors when a single dose of radiation is used. For DSBs, the larger volume of 31.5 ml is used in order to reduce the effect of analyzing profiles in the transition region between first-order elution and the subsequent slower elution.

E8. Radioprotective Drugs and Radiation Treatments

For the *in vitro* studies, drugs are dissolved in growth medium immediately prior to use and sterilized by filtration. For the *in vivo* studies, the drugs are dissolved in physiological saline immediately prior to injection i.p. All irradiations are performed using a ^{137}Cs-irradiator with a dose rate of 5 Gy/min.

E9. Gut Microcolony Assay

The principles of this assay have been discussed in detail elsewhere (33). Briefly, mice are irradiated with up to 26 Gy of X-rays. Jejunal cross-sections obtained from animals both before and after irradiation, either with or without a 30 min pretreatment with the radioprotective agent, are prepared for histological examination 3.5 days after irradiation. The number of regenerating crypts are estimated by eye using a light microscope. The numbers are then converted to the number of surviving cells by applying a Poisson distribution correction function.

E10. Spleen-colony assay

This assay was developed by Till and McCulloch (45) for examining the effects of radiation on bone-marrow stem cells. Mice (8 per group) are irradiated with graded doses of radiation: for the placebo control animals this is usually in the vicinity

of 6-8 Gy (in 0.5 Gy increments) and for the drug-treated animals is appropriately higher. 8 days after irradiation the spleens are removed from the animals and fixed in Bouin's solution. The number of gross surface spleen colonies are then counted.

F. LITERATURE CITED

1. Smoluk, G.D., Fahey, R.C., Calabro-Jones, P.M., Aguilera, J.A., and Ward, J.F. (1988) Radioprotection of cells in culture by WR-2721 and derivatives: Protection correlates with intracellular WR-1065. Cancer Res. in press.

2. Sweeney, T.R. (1979) A Survey of Compounds from the Antiradiation Drug Development Program of the U.S. Army Medical Research and Development Command. Washington, D.C.

3. Davidson, D.E., Grenan, M.M, and Sweeney, T.R. (1980) Biological characteristics of some improved radioprotectors. In Radiation Sensitizers: Their Use in the Clinical Management of Cancer. (edited by L.W. Brady) New York, Masson Publishing, pp. 309-320.

4. Klayman, D.L. and Copeland, E.S. (1982) Radioprotective agents. Kirk-Othmer: Encyclopedia of Chemical Technology 3rd Edition, (edited by M. Grayson) New York, John Wiley & Sons Volume 19, pp. 801-832.

5. Livesey, J.C. and Reed, D.J. (1987) Chemical protection against ionizing radiation. In: Advances in Radiation Biology Volume 13. (edited by J.T. Lett) San Diego, Academic Press, pp. 285-354.

6. Painter, R.B. (1980) The role of DNA damage and repair in cell killing induced by ionizing radiation. In: Radiation Biology in Cancer Research (edited by R.E. Meyn and H.R. Withers) New York, Raven Press, pp. 59-70.

7. Elkind, M.M. (1985) DNA damage and cell killing. Cause and effect? Cancer 56:2351-2363.

8. Murray, D. (1987) Annual Report "Biological Evaluation of Radioprotective Drugs".

9. Murray, D., vanAnkeren, S., Milas, L. and Meyn, R.E. (1988) Radioprotective action of WR-1065 on radiation-induced DNA strand breaks in cultured chinese hamster ovary cells. Radiation Research 113:155-170.

10. Murray, D., Altschuler, E., Kerr, M., vanAnkeren, S.C., Meyn, R.E., and Milas, L. (1988) Radioprotection of cultured Chinese hamster ovary cells by WR-255591. International Journal of Radiation Biology, in press.

11. Ward, J.F. (1983) Chemical Aspects of DNA Radioprotection. In: Radioprotectors and Anticarcinogens (edited by O.F. Nygaard and M.G. Simic) New York, Academic Press, pp. 73-85.

12. Purdie, J.W. (1979) A comparative study of the radioprotective effects of cysteamine, WR-2721, and WR-1065

in cultured human cells. Radiat. Res. 77, 303-311.

13. Livesey, J.C., Rasey, J.S., Vertrees, S., Freeman, L.M., Magee, S., Nelson, N.J., Chin, L., Grunbaum, Z., and Krohn, K.A. (1988) In vitro metabolism of the phosphorothioate radioprotectors WR-2721 and WR-3689. Pharmacology and Therapeutics in press.

14. Calabro-Jones, P.M., Fahey, R.C., Smoluk, G.D., and Ward, J.F. (1985) Alkaline phosphatase promotes radioprotection and accumulation of WR-1065 in V79-171 cells incubated in medium containing WR-2721. Int. J. Radiat. Biol. 47:23-27.

15. Calabro-Jones, P.M., Aguilera, J.A., Ward, J.F., Smoluk, G.D., and Fahey, R.C. (1988) Uptake of WR-2721 derivatives by cells in culture: WR-1065 is the transported form of the drug. Cancer Res. in press.

16. Kohn, K.W., Ewig, R.A.G., Erickson, L.C., and Zwelling, L.A. (1981) Measurement of strand breaks and cross-links by alkaline elution. In DNA Repair: A Laboratory Manual of Research Procedures (edited by E.C. Friedberg and P.C. Hanawalt) New York, Marcel Dekker, Inc., pp. 379-402.

17. Murray, D., vanAnkeren, S.C., Milas, L. and Meyn, R.E. (1988) The role of modification of DNA damage in the radioprotective action of aminothiols. Proceedings of the Second International Conference on Anticarcinogenesis. in press.

18. Held, K.D., Bren, G.D. and Melder, D.C. (1986) Interactions of radioprotectors and oxygen in cultured mammalian cells. II. Effects of dithiothreitol on radiation-induced DNA damage and comparison with cell survival. Radiat. Res. 108:296-306.

19. Smoluk, G.D., Fahey, R.C., and Ward, J.F. (1986) Equilibrium dialysis studies of the binding of radioprotector compounds to DNA. Radiat. Res. 107:194-205.

20. Sigdestad, C.P., Treacy, S.H., Knapp, L.A., and Grdina, D.J. (1987) The effect of 2-[(aminopropyl)amino] ethanethiol (WR-1065) on radiation induced DNA double strand damage and repair in V79 cells. British Journal of Cancer 55:477-482.

21. Radford, I.R. (1986) Effect of radiomodifying agents on the ratios of x-ray-induced lesions in cellular DNA: Use in lethal lesion determination. Int. J. Radiat. Biol. 49:621-637.

22. Radford, I.R. (1986) Evidence for a general relationship between the induced level of DNA double-strand breakage and cell-killing after x-irradiation of mammalian cells. Int. J. Radiat. Biol. 49:611-620.

23. Zheng, S., Newton, G.L., Gonick, G., Fahey, R.C., and Ward, J.F. (1988) Radioprotection of DNA by thiols: Relationship between the net charge on a thiol and its ability to protect DNA. Radiation Research in press.

24. Elkind, M.M. (1979) DNA repair and cell repair: Are they related? Int. J. Radiat. Oncol. Biol. Phys. 5:1089-1094.

25. Brown, P.E. (1967) Mechanisms of action of aminothiol radioprotectors. Nature 213:363-364.

26. Sawada, S. and Okada, S. (1970) Cysteamine, cystamine, and single-strand breaks of DNA in cultured mammalian cells. Radiat. Res. 44:116-132.

27. Grdina, D.J. and Nagy, B. (1986) The effect of 2-[(aminopropyl)amino] ethanethiol (WR-1065) on radiation-induced DNA damage and repair and cell progression in V79 cells. Br. J. Cancer, 54:933-941.

28. Utsumi, H. and Elkind, M.M. (1986) Potentially lethal damage, deficient repair in x-ray-sensitive caffeine-responsive Chinese hamster cells. Radiat. Res. 107:95-106.

29. Murray, D. and Meyn, R.E. (1987) Differential repair of γ-ray-induced DNA strand breaks by various cellular subpopulations of mouse jejunal epithelium and bone marrow in vivo. Radiat. Res. 109:153-164.

30. Murray, D., Jenkins, W.T., and Meyn, R.E. (1984) The efficiency of DNA strand-break repair in two fibrosarcoma tumors and in normal tissues of mice irradiated in vivo with X-rays. Radiat. Res. 100:171-181.

31. Murray, D., Milas, L., and Meyn, R.E. (1988) Radioprotection of mouse jejunum by WR-2721 and WR-1065: Effects on DNA strand-break induction and rejoining. Radiat. Res. 114:268-280.

32. Murray, D., Milas, L., and Meyn, R.E. (1988) Protection by WR-3689 of mouse jejunum from the cytotoxic and DNA damaging effects of γ-rays. British Journal of Cancer (submitted).

33. Withers, H.R. and Elkind, M.M. (1970) Microcolony survival assay for cells of mouse intestinal mucosa exposed to radiation. Int. J. Radiat. Biol. 17:261-267.

34. Brown, D.Q., Yuhas, J.M., Mackenzie, L.J., Graham, W.J., III, Pittock, J.W., III (1984) Differential radioprotection of normal tisseus by hydrophilic chemical protectors. Int. J. Radiat. Oncol. Biol. Phys. 10:1581-1584.

35. Rasey, J.S., Krohn, K.A., Magee, S., Nelson, N., and Chin,

L. (1986) Comparison of the protective effects of three phosphorothioate radioprotector in the RIF-1 tumor. Radiat. Res. 108:167-175.

36. Rasey, J.S., Krohn, K.A., Grunbaum, Z., Spence, A.M., Menard, T.W. and Wade, R.A. (1986) Synthesis, biodistribution, and autoradiography of radiolabeled S-2-(3-methylaminopropylamino)ethylphosphorothioic acid (WR-3689). Radiat. Res. 106:366-379.

37. Milas, L., Hunter, N., Reid, B.O., and Thames, H.D. Jr. (1982) Protective effects of S-2-(3-aminopropylamino)ethylphosphorothioic acid against radiation damage of normal tissues and a fibrosarcoma in mice. Cancer Res. 42:1888-1897.

38. Rasey, J.S., Nelson, N.J., Mahler, P., Anderson, K., Krohn, K.A., and Menard, T. (1984) Radioprotection of normal tissue against gamma rays and cyclotron neutrons with WR-2721: LD_{50} studies and ^{35}S-WR-2721 biodistribution. Radiat. Res. 97:598-607.

39. Travis, E.L. (1984) The oxygen dependence of protection by aminothiols: Implications for normal tissues and solid tumors. Int. J. Radiat. Oncol. Biol. Phys. 10:1495-1501.

40. Hendry, J.H., Potten, C.S., and Roberts, N.P. (1983) The gastrointestinal syndrome and mucosal clonogenic cells: Relationships between target cell sensitivities, LD_{50} and cell survival, and their modification by antibiotics. Radiat. Res. 96:100-112.

41. Hanson, W.R. (1987) Radiation protection of murine intestine by WR-2721, 16, 16-Dimethyl Prostaglandin E_2, and the combination of both agents. Radiat. Res. 111:361-373.

42. Ward, J.F. (1986) Mechanisms of DNA repair and their potential modification for radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. 12:1027-1032.

43. Meyn, R.E. and Jenkins, W.T. (1983) Variation in normal and tumor tissue sensitivity of mice to ionizing radiation-induced DNA strand breaks in vivo. Cancer Res. 43:5668-5673.

44. Murray, D, Meyn, R.E., and vanAnkeren, S.C. (1988) Variations in the spectrum of lesions produced in the DNA of cells from mouse tissues after exposure to γ-rays in air-breathing or in artificially anoxic animals. Int. J. Radiat. Biol. in press.

45. Till, J.E. and McCulloch, E.A. (1963) Early repair processes in marrow cells irradiated and proliferating in vivo. Radiat. Res. 18:96-105.

46. Bowman, G.T., Clement, J.J., Davidson, D.E., Jr., Eswarakrishnan, V. Field, L., Hoch, J.M., Musallam, H.A., Pick, R.O., Ravichandran, R., and Srivastava, P.K. (1986) Potential antiradiation drugs containing no nitrogen, and related compounds. Chem-Biol. Interactions 57:161-174.

47. Bradley, M.O. and Kohn, K.W. (1979) X-ray induced DNA double strand break production and repair in mammalian cells as measured by neutral filter elution. Nucleic Acids Res., 7:793-804.

48. Murray, D., vanAnkeren, S.C. and Meyn, R.E. (1987) Applicability of the alkaline elution procedure as modified for the measurement of DNA damge and its repair in nonradioactively-labeled cells. Anal. Biochem. 160:149-159.

SECTION G. FIGURES AND TABLES

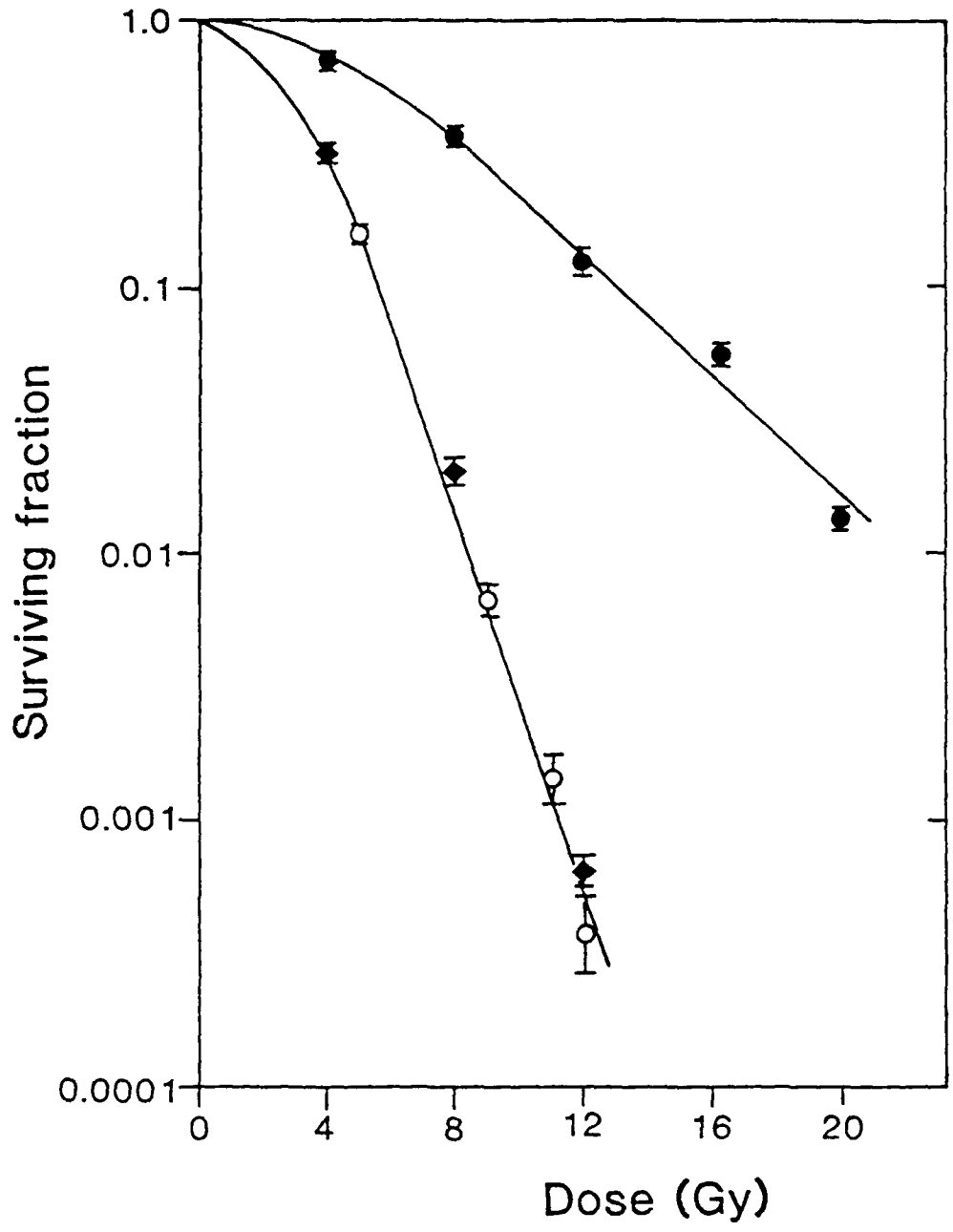


FIGURE 1. Effect of 6 mM WR-255591 on the survival of CHO cells irradiated on ice. The cells were either irradiated following a 30-min pretreatment with WR-255591 (●) or were irradiated in the absence of the drug, warmed to 37°C, and then incubated for 60-min in the presence of WR-255591 (◆). The control cells (○) were irradiated on ice in drug-free medium.

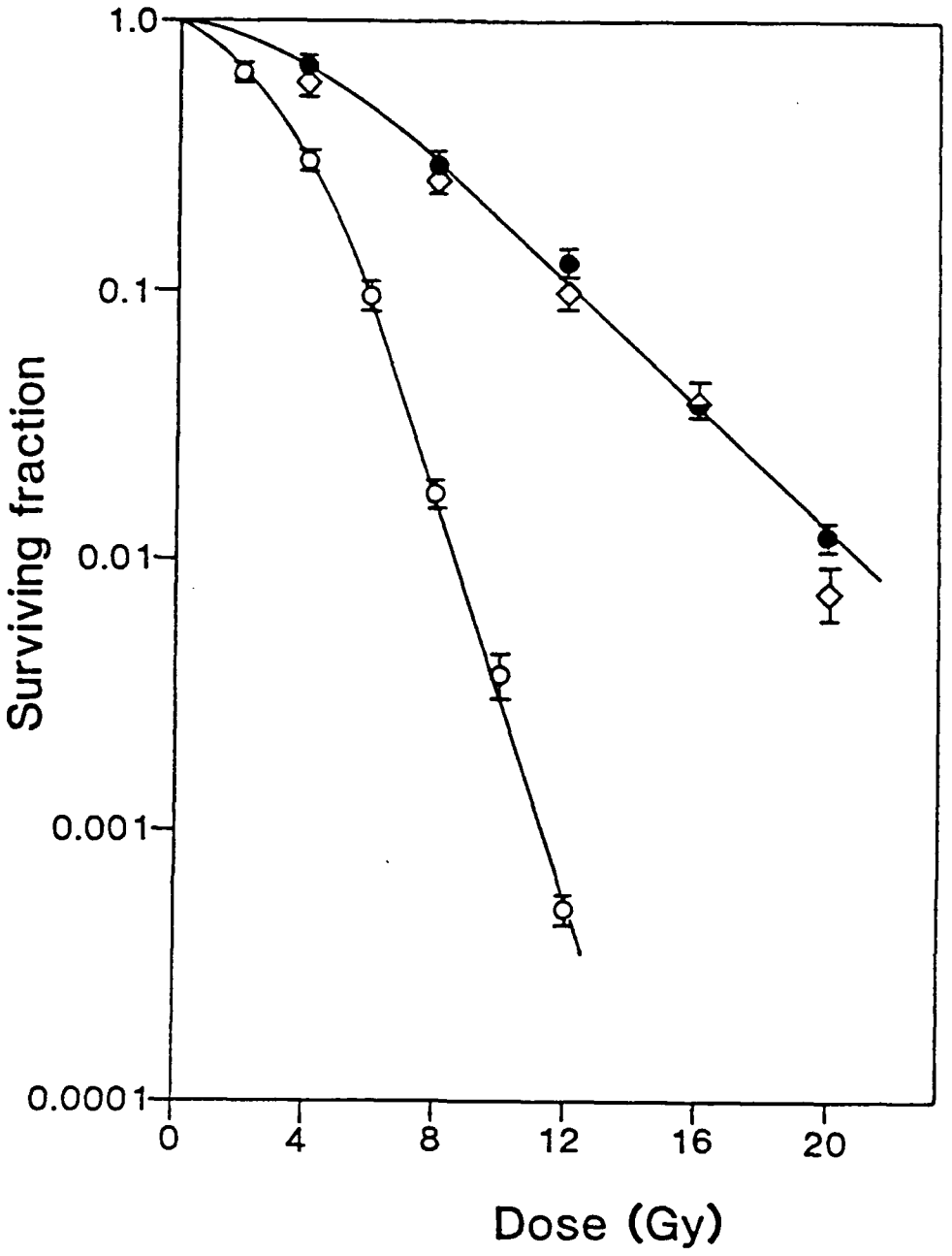


FIGURE 2. Effect of 6 mM WR-255591 on the survival of CHO cells irradiated at 37°C. Cells were treated with WR-255591 for 30-min prior to and during the irradiation; they were then either trypsinized and plated for survival (●) or were incubated for a further 60-min at 37°C in the presence of the drug before trypsinization and plating (◇). The control cells (○) were irradiated in drug-free growth medium at 37°C.

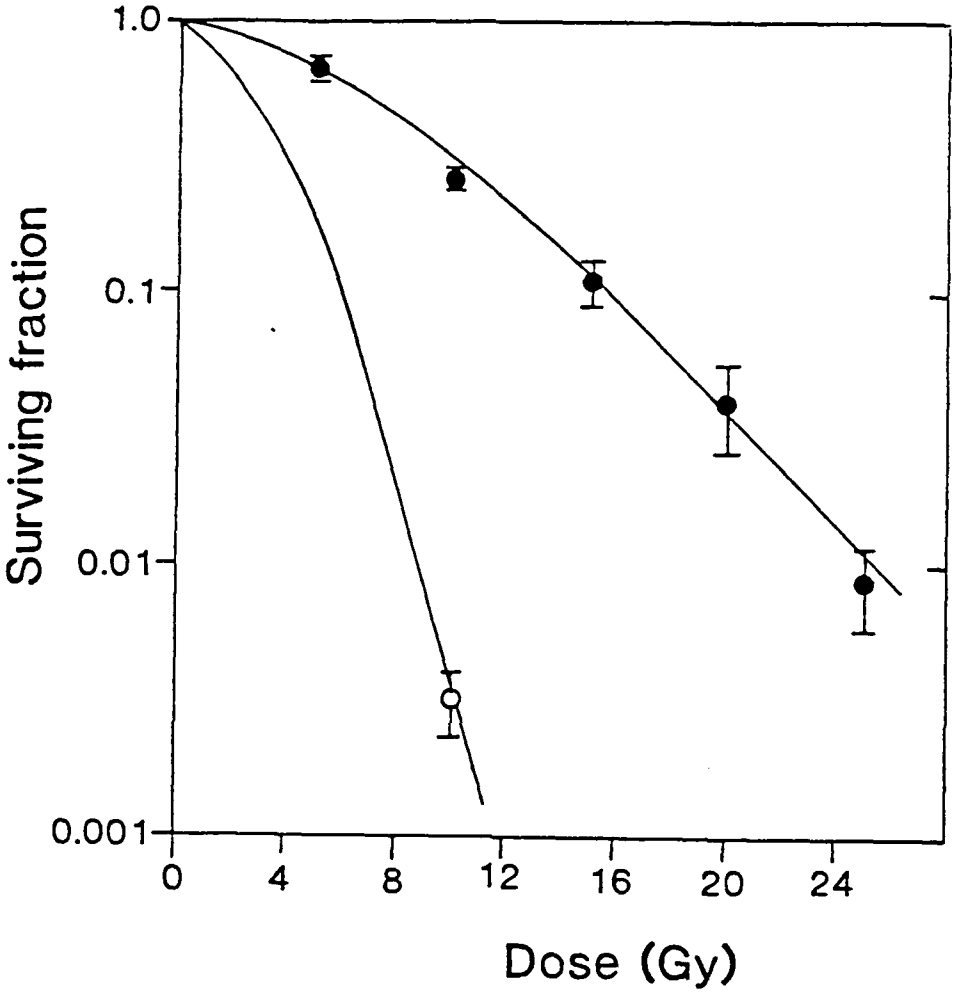


FIGURE 3. Effect of 6 mM WR-151326 on the γ-ray survival of CHO cells irradiated at 37°C. Cells were treated with WR-151326 (●) for 0.5-h prior to irradiation. The control cells (○) were irradiated in drug-free growth medium at 37°C; the solid line shows a cumulation of control data, and the single point represents the 10 Gy data for control cells irradiated along with the WR-151326-treated cells.

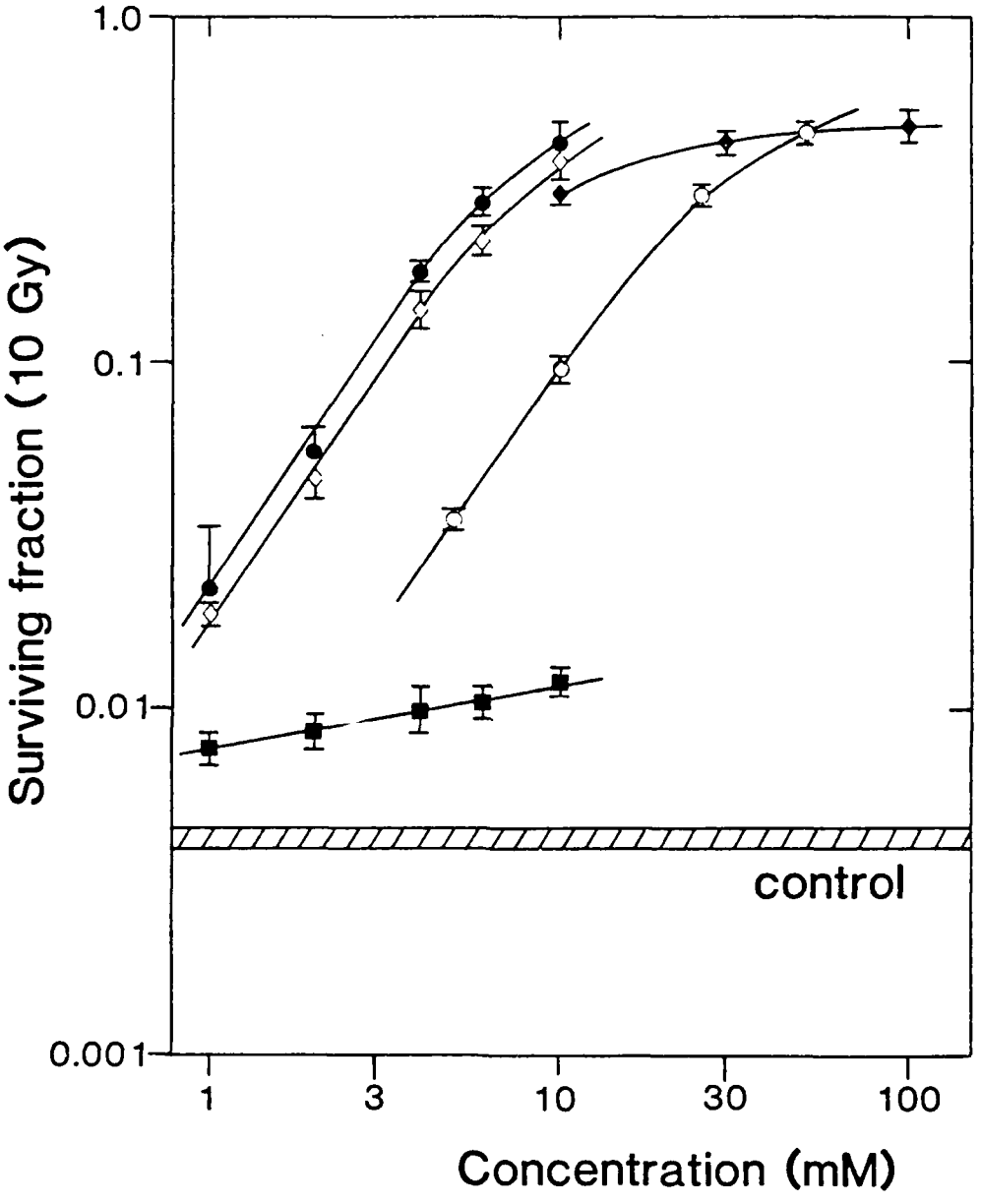


FIGURE 4. Effect of increasing concentrations of WR-1065 (●), WR-255591 (◇), WR-3689 (■), DTT (○) or cysteamine (◆) on the survival of CHO cells irradiated with 10 Gy of γ-rays at 37°C. The drug treatments were for 30-min at 37°C in all cases.

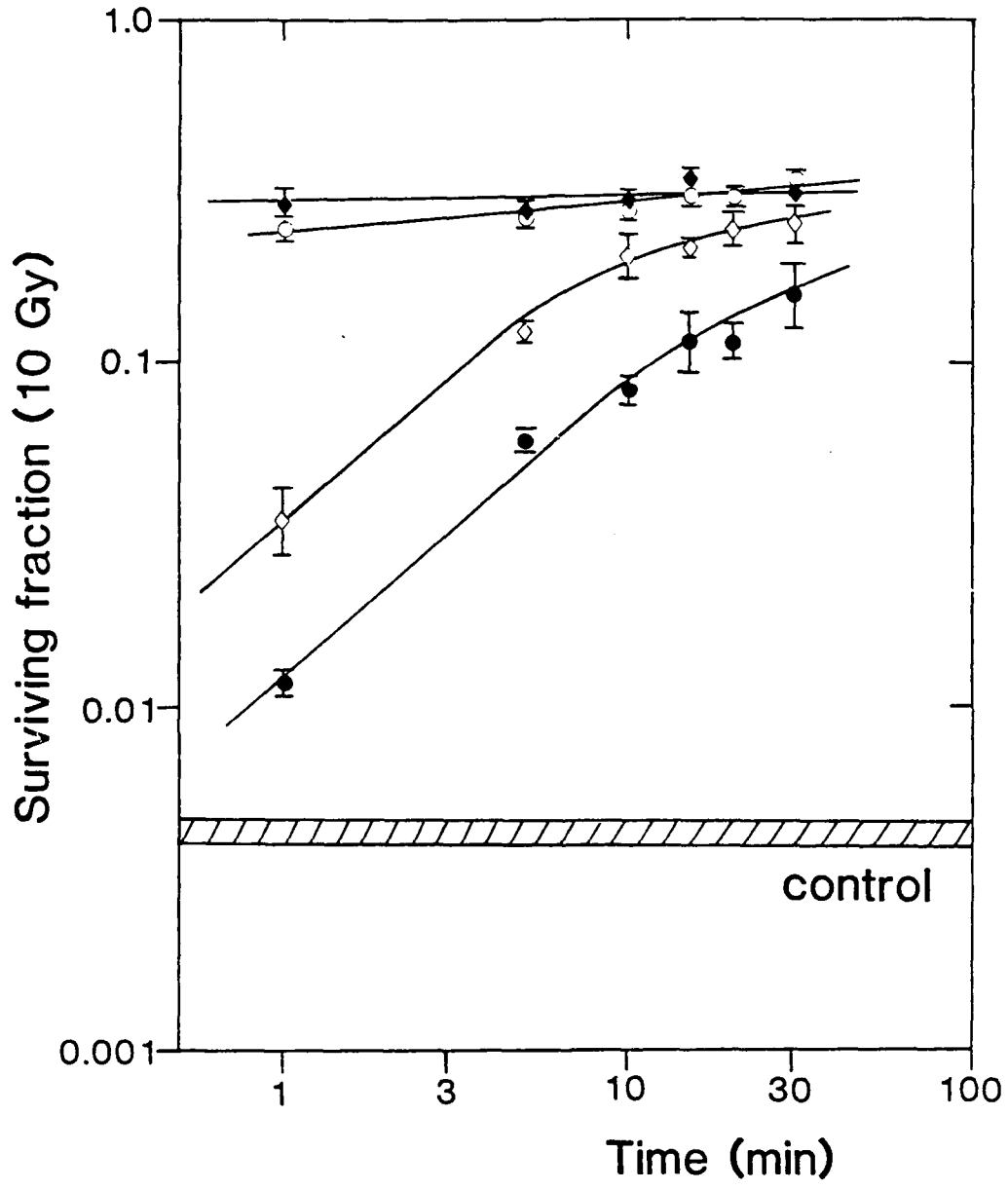


FIGURE 5. Effect of varying lengths of exposure to thiols on radioprotection. CHO cells were treated with either 4 mM WR-1065 (●), 6 mM WR-255591 (◇), 10 mM cysteamine (◆) or 25 mM DTT (○) for various times and then irradiated with 10 Gy of γ-rays at 37°C.

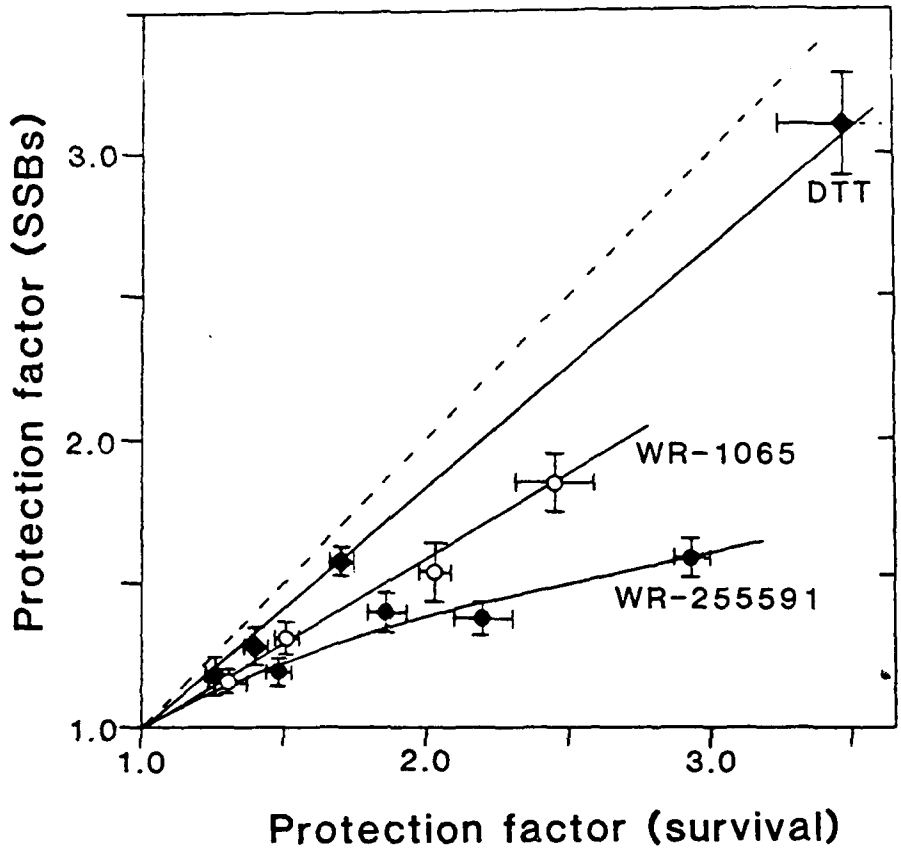


FIGURE 6. Correlation curves showing the relationship between the protection factor for DNA single-strand breaks and cell survival. The drugs investigated were WR-1065 (○), DTT (◆), and WR-255591 (●); cells were irradiated at 37°C either in growth medium or following a 30-min incubation at 37°C with increasing concentrations of each drug.

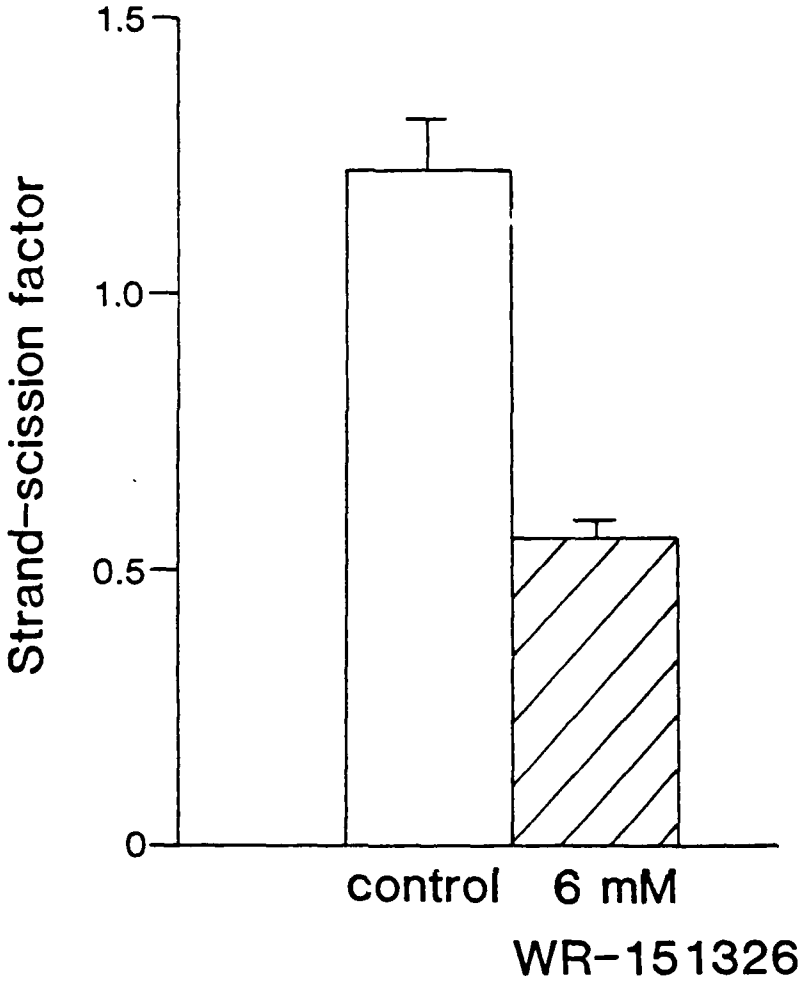


FIGURE 7. Effect of WR-151326 on the yield of γ-ray-induced DNA single-strand breaks. CHO cells were irradiated with 5 Gy on ice either in growth medium (control) or following a 30-min treatment at 37°C with 6 mM WR-151326.

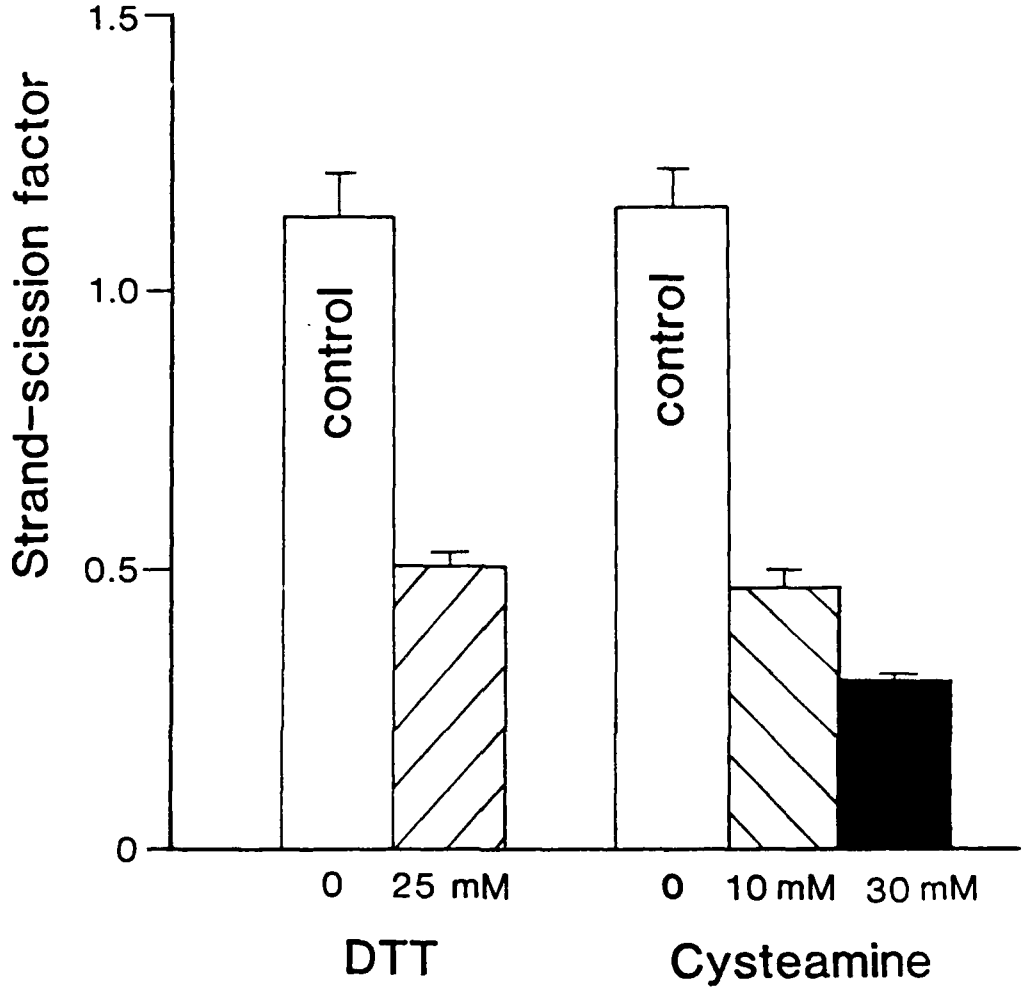


FIGURE 8. Effect of cysteamine and DTT on the yield of γ-ray-induced DNA single-strand breaks. CHO cells were irradiated with 5 Gy on ice either in growth medium (controls) or following a 30-min incubation at 37°C with either 25 mM DTT or 10 or 30 mM cysteamine. The drug was present for 30-min prior to and throughout the duration of the irradiation.

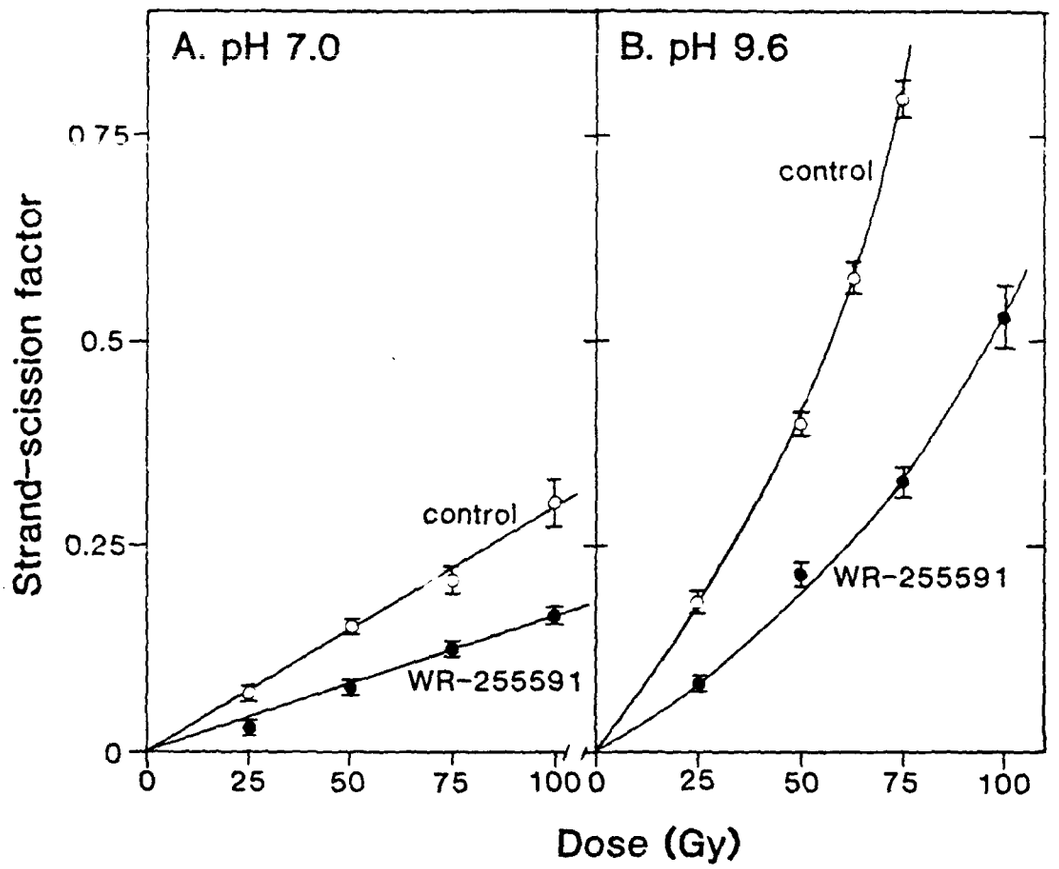


FIGURE 9. Effect of WR-255591 on the yield of γ-ray-induced DNA double-strand breaks. CHO cells were irradiated on ice either in growth medium (○) or following a 30-min incubation with 6 mM WR-255591 (●). The drug was present for 30-min prior to and throughout the duration of the irradiation. Double-strand breaks were assayed either at (A) pH 7.0.; or (B) pH 9.6.

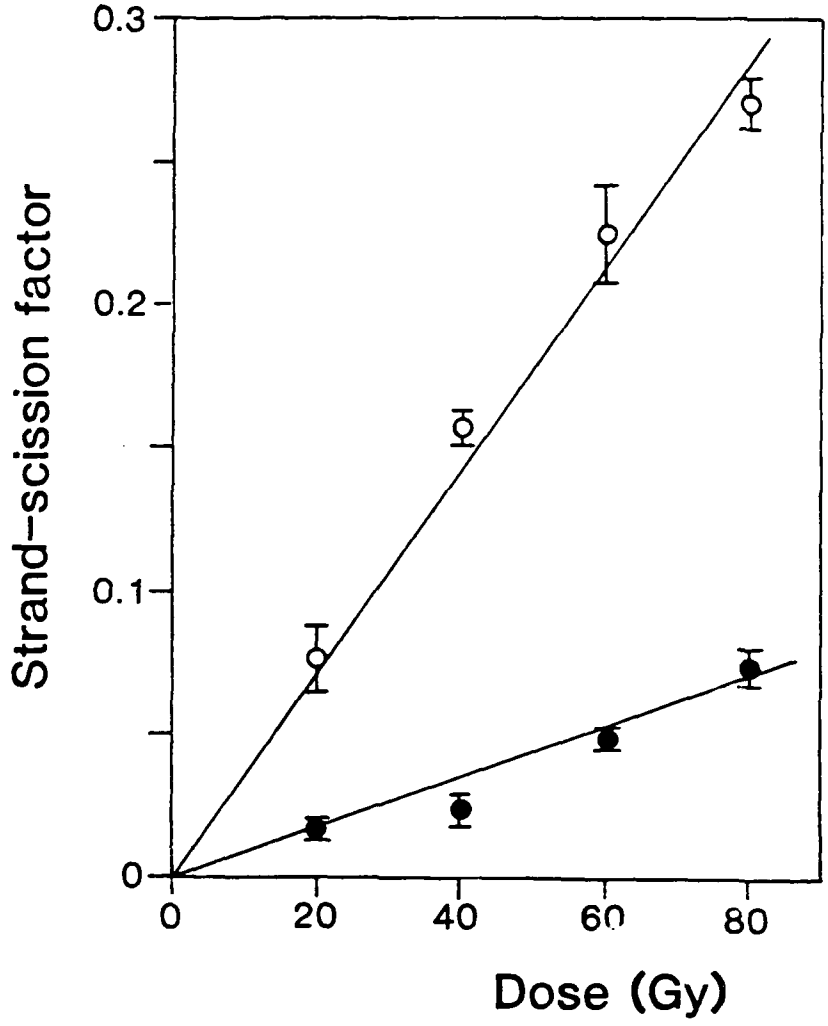


FIGURE 10. Effect of cysteamine on the yield of γ-ray-induced DNA double-strand breaks. CHO cells were irradiated on ice either in growth medium (○) or following a 30-min incubation with 30 mM cysteamine (●). The drug was present for 30-min prior to and throughout the duration of the irradiation. DNA double-strand breaks were assayed at pH 7.0.

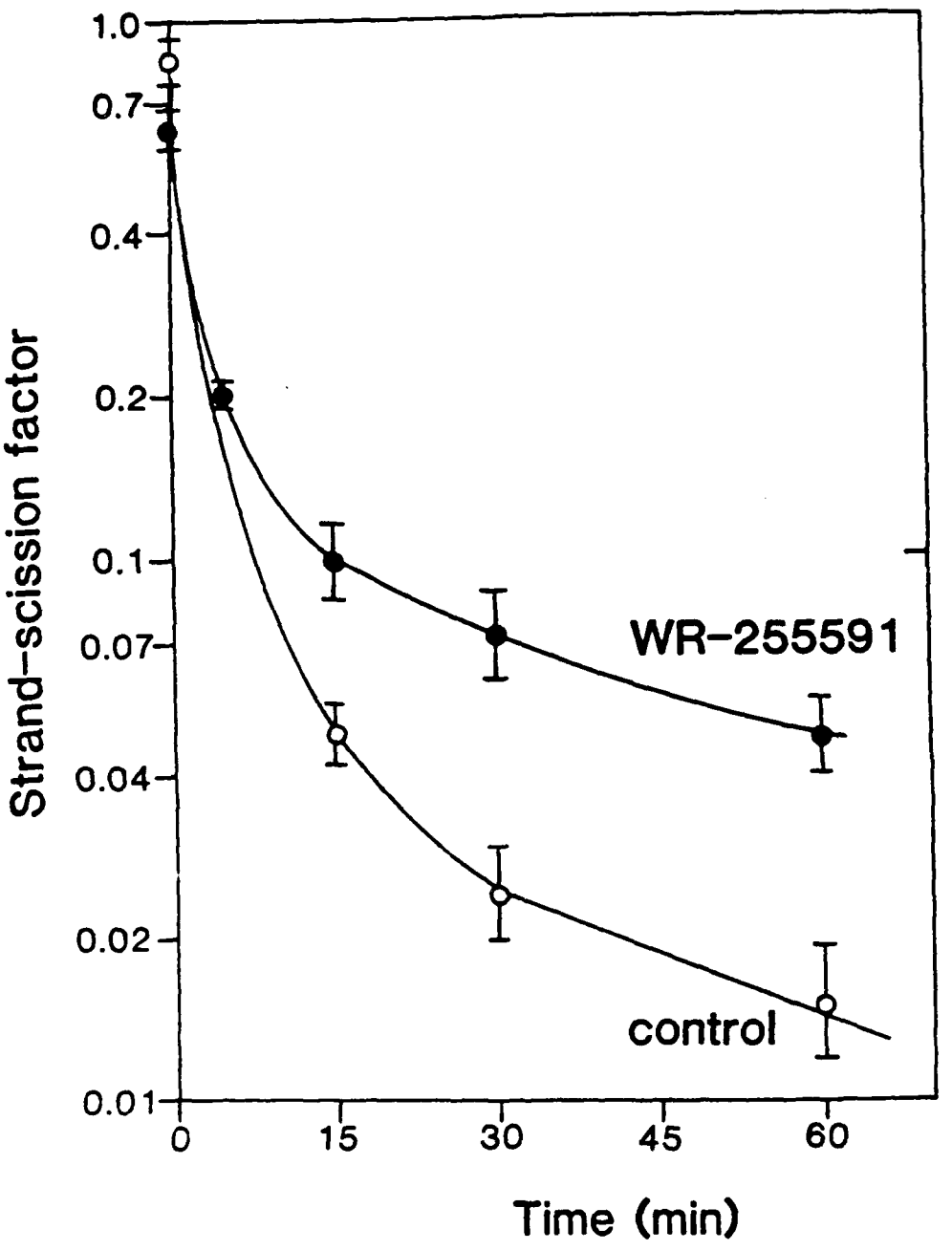


FIGURE 11. Effect of pre- and post-irradiation incubation with 6 mM WR-255591 on the rejoining of DNA single-strand breaks: CHO cells were irradiated at 37°C with 5 Gy of γ-rays.
(○) controls, no drug treatment.
(●) cells were irradiated immediately following a 30-min pretreatment with 6 mM WR-255591, after which they were allowed to repair at 37°C. Since the drug was not removed, the cells were therefore exposed to the drug during their repair incubation.

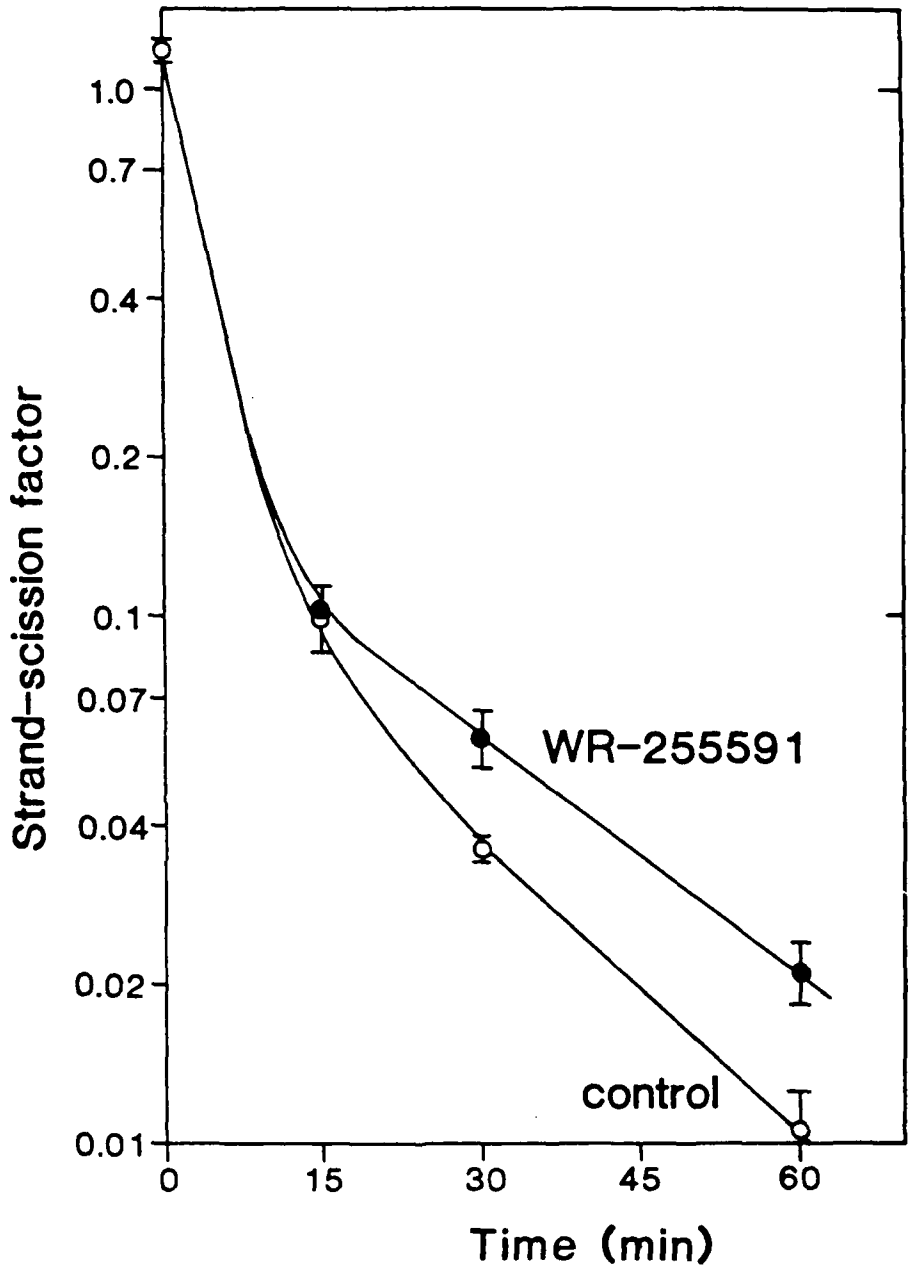


FIGURE 12. Effect on single-strand break rejoining when cells were exposed to 6 mM WR-255591 only during their repair incubation. The cells were irradiated on ice with 5 Gy. Both catalase and desferal were included in the media.

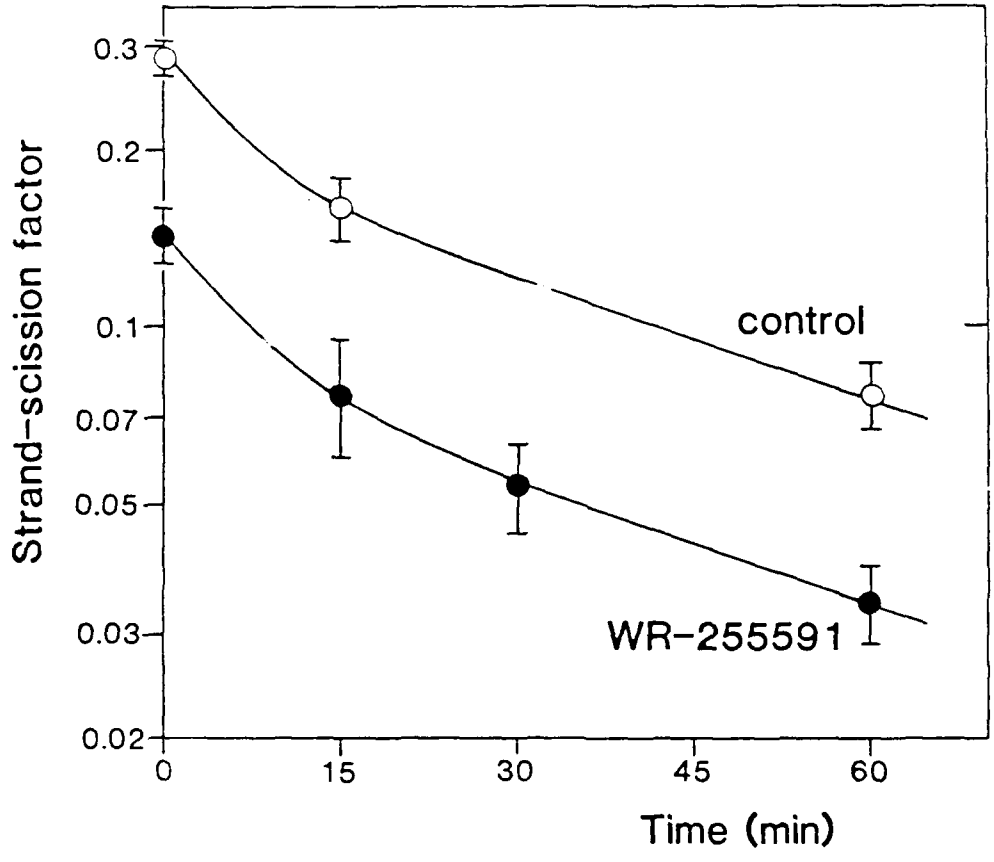


FIGURE 13. Effect of WR-255591 on the rejoining of DNA double-strand breaks assayed at pH 7.0 in CHO cells. Cells were irradiated on ice with 100 Gy. The experimental protocols were as follows:

(○) control cells, no drug treatment.

(●) pre-irradiation and post-irradiation incubation with 6 mM WR-255591.

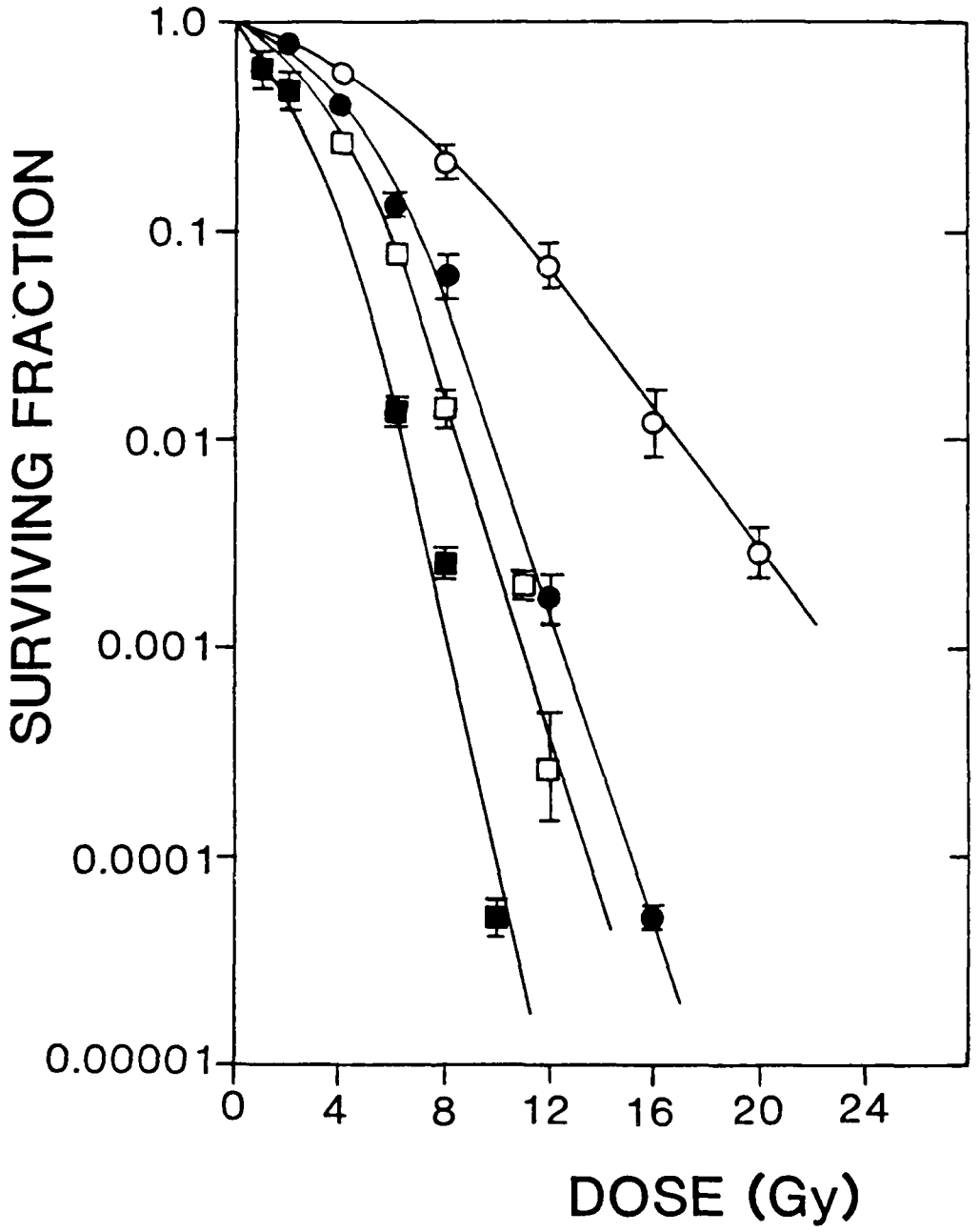


FIGURE 14. Effect of immediate post-irradiation treatment with hypertonic salt on the survival of log-phase CHO cells. Cells were irradiated on ice either in the presence (●) or absence (■) of 4 mM WR-1065 (30-min pretreatment at 37°C) and then treated with hypertonic salt for 20-min. Also shown are survival curves for cells treated in identical fashion, i.e. with (○) or without (□) 4 mM WR-1065, except that they were not subsequently treated with hypertonic salt.

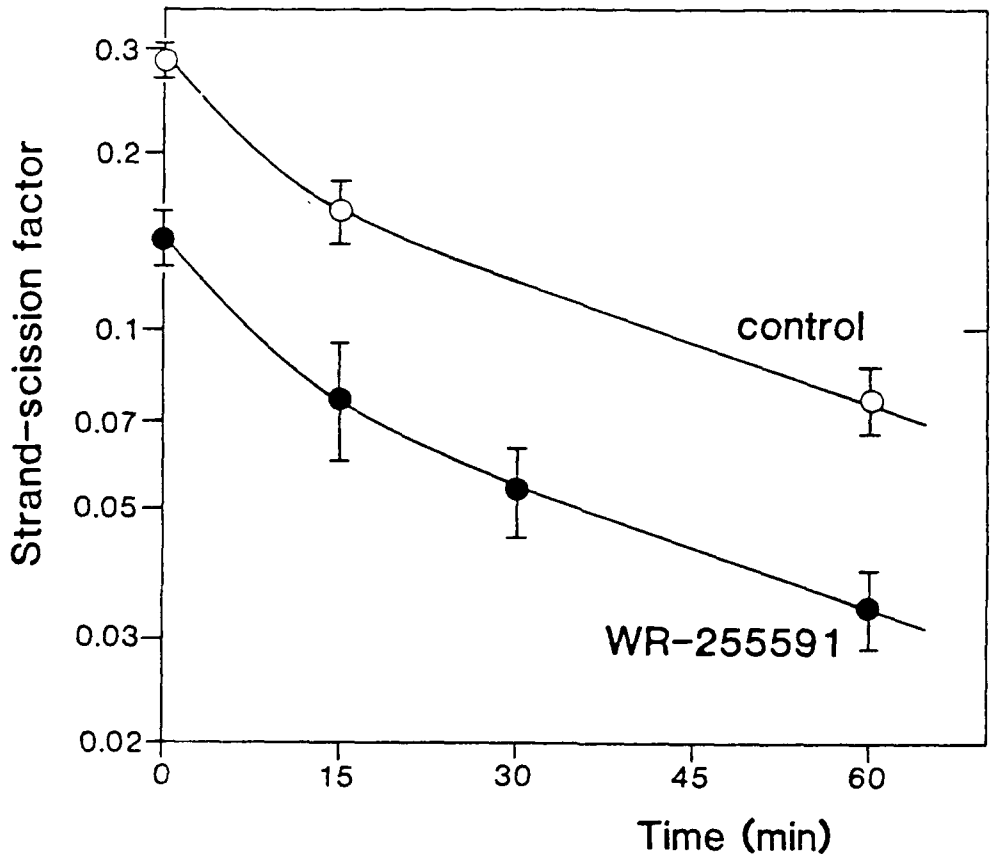


FIGURE 13. Effect of WR-255591 on the rejoining of DNA double-strand breaks assayed at pH 7.0 in CHO cells. Cells were irradiated on ice with 100 Gy. The experimental protocols were as follows:

(○) control cells, no drug treatment.

(●) pre-irradiation and post-irradiation incubation with 6 mM WR-255591.

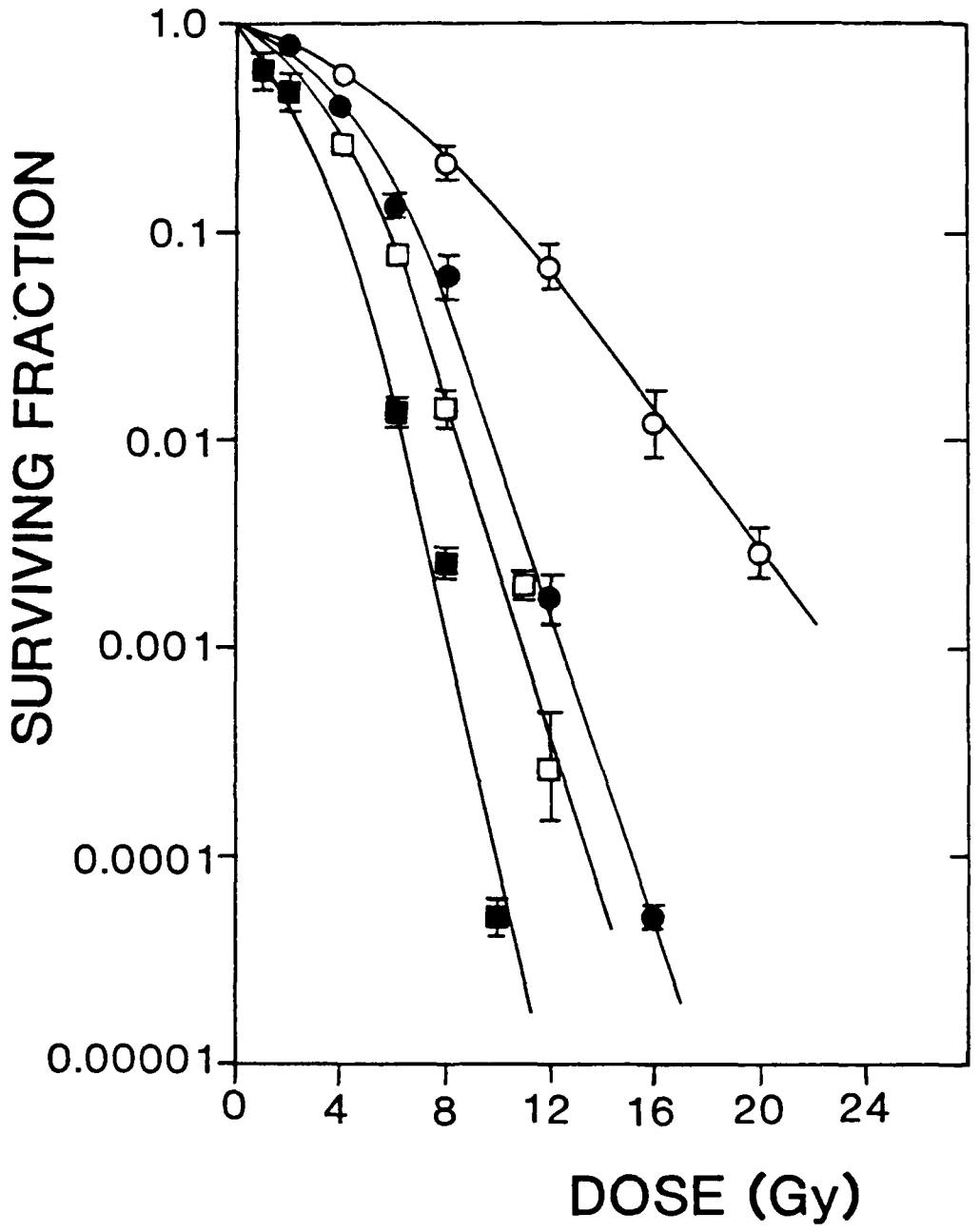


FIGURE 14. Effect of immediate post-irradiation treatment with hypertonic salt on the survival of log-phase CHO cells. Cells were irradiated on ice either in the presence (●) or absence (■) of 4 mM WR-1065 (30-min pretreatment at 37°C) and then treated with hypertonic salt for 20-min. Also shown are survival curves for cells treated in identical fashion, i.e. with (○) or without (□) 4 mM WR-1065, except that they were not subsequently treated with hypertonic salt.

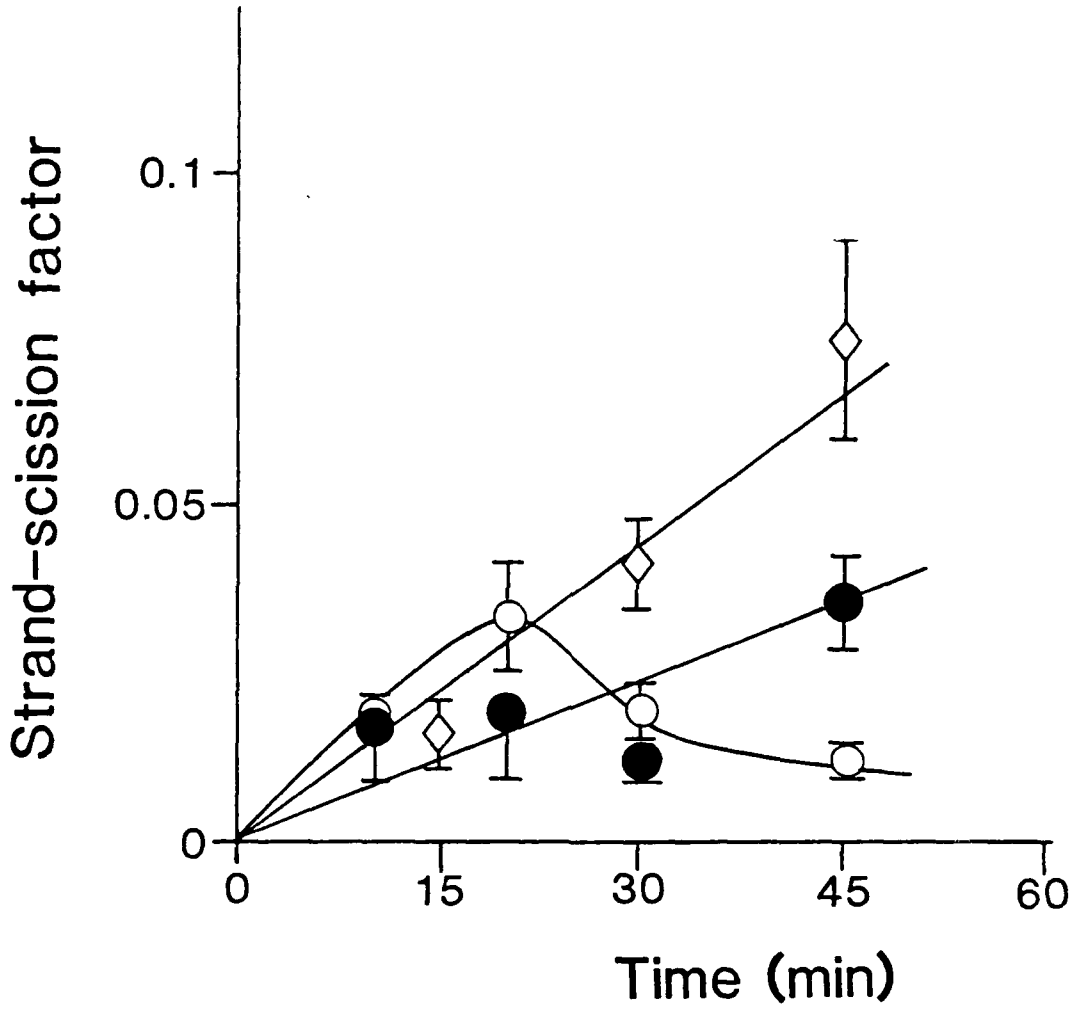


FIGURE 15. Induction of DNA single-strand breaks in cells exposed to various thiols. CHO cells were treated at 37°C for various times with either 10 mM WR-255591 (◇), 10 mM WR-1065 (●) or 50 mM DTT (○).

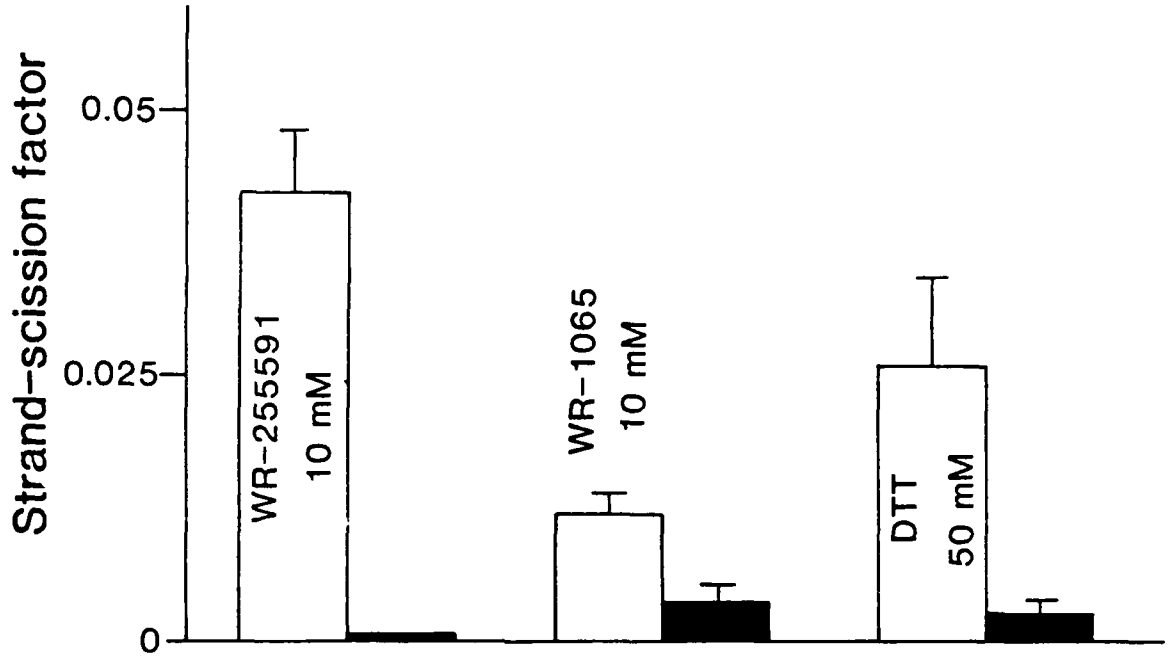


FIGURE 16. Effect of orthophenanthroline on the induction of DNA single-strand breaks by thiol compounds. Cells were treated at 37°C for 30-min with either 10 mM WR-255591, 10 mM WR-1065 or 50 mM DTT either with (shaded bars) or without (open bars) 10 μM orthophenanthroline.

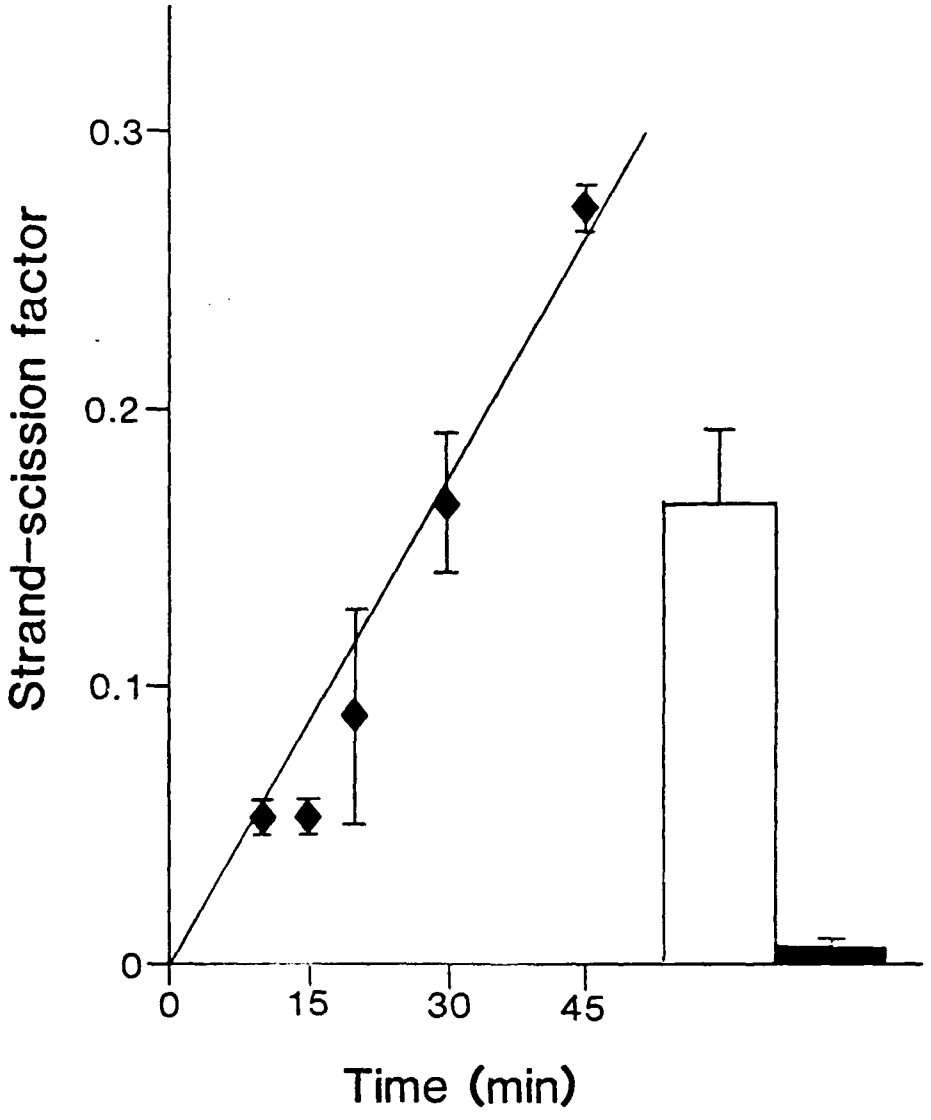


FIGURE 17. Time course of and effect of orthophenanthroline on cysteamine-induced DNA single-strand breakage. All drug treatments were with 100 mM cysteamine at 37°C. The open and solid bars indicate the level of breaks after a 30-min treatment without or with 10 μM orthophenanthroline, respectively.

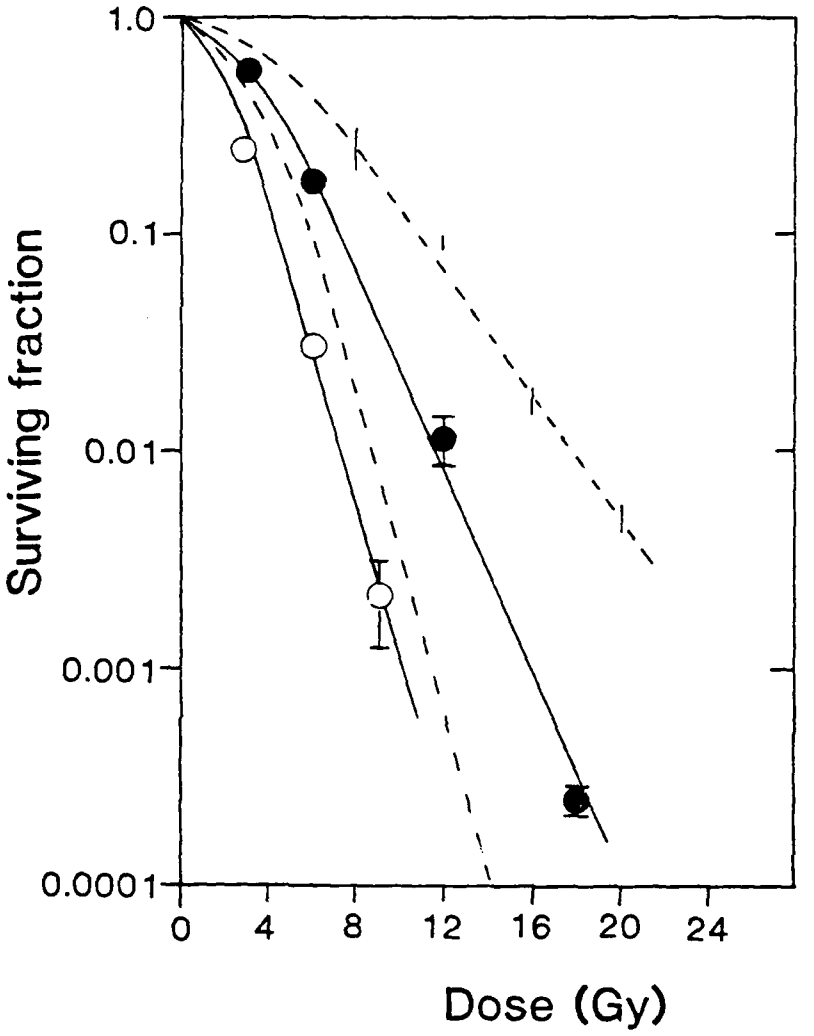


FIGURE 18. Effect of glutathione depletion by buthionine sulfoximine (BSO) on radioprotection by WR-1065. The cells were treated with BSO (0.5 mM) for 24 h. The cells were then incubated either in medium (○) or with 4 mM WR-1065 (●) for 30 min prior to irradiation. The dashed lines show survival curves for cells that were not exposed to BSO but were otherwise treated in identical fashion.

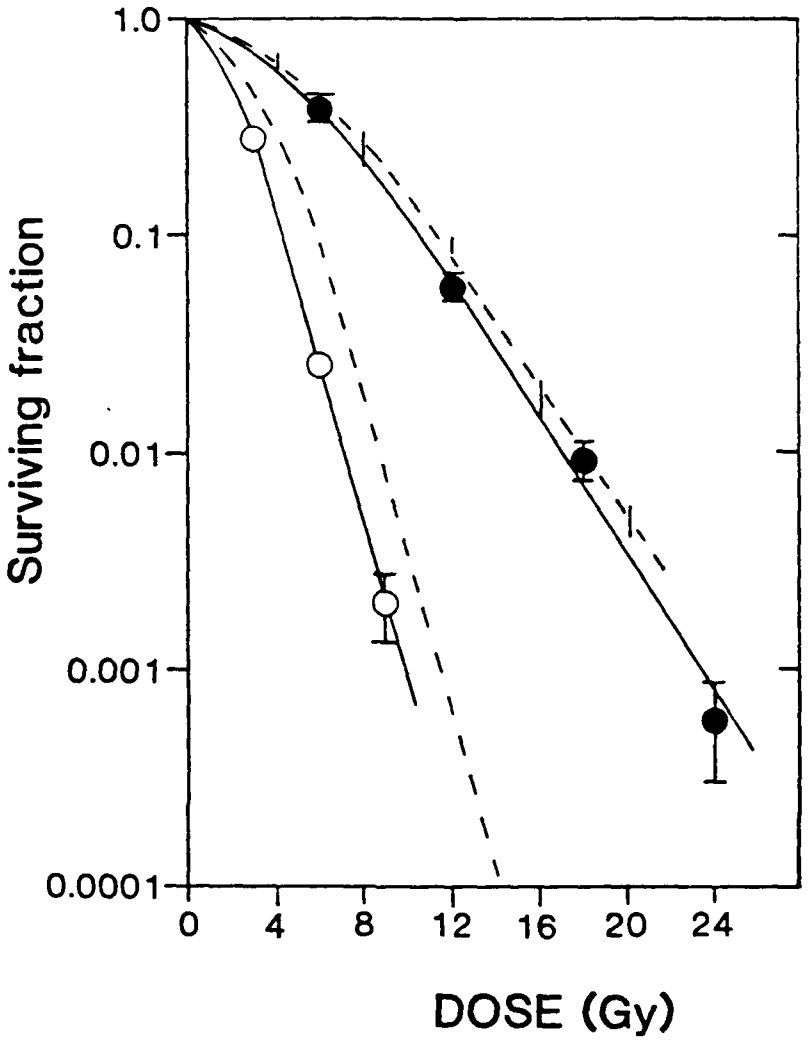


FIGURE 19. Effect of polyamine depletion by γ-difluoromethylornithine (DFMO) on radioprotection by WR-1065. The cells were exposed to DFMO (1 mM) for 48 h followed by a 30-min treatment with either growth medium (○) or 4 mM WR-1065 (●) followed by irradiation. The dashed lines show survival curves for cells that were not exposed to DFMO but were otherwise treated in identical fashion.

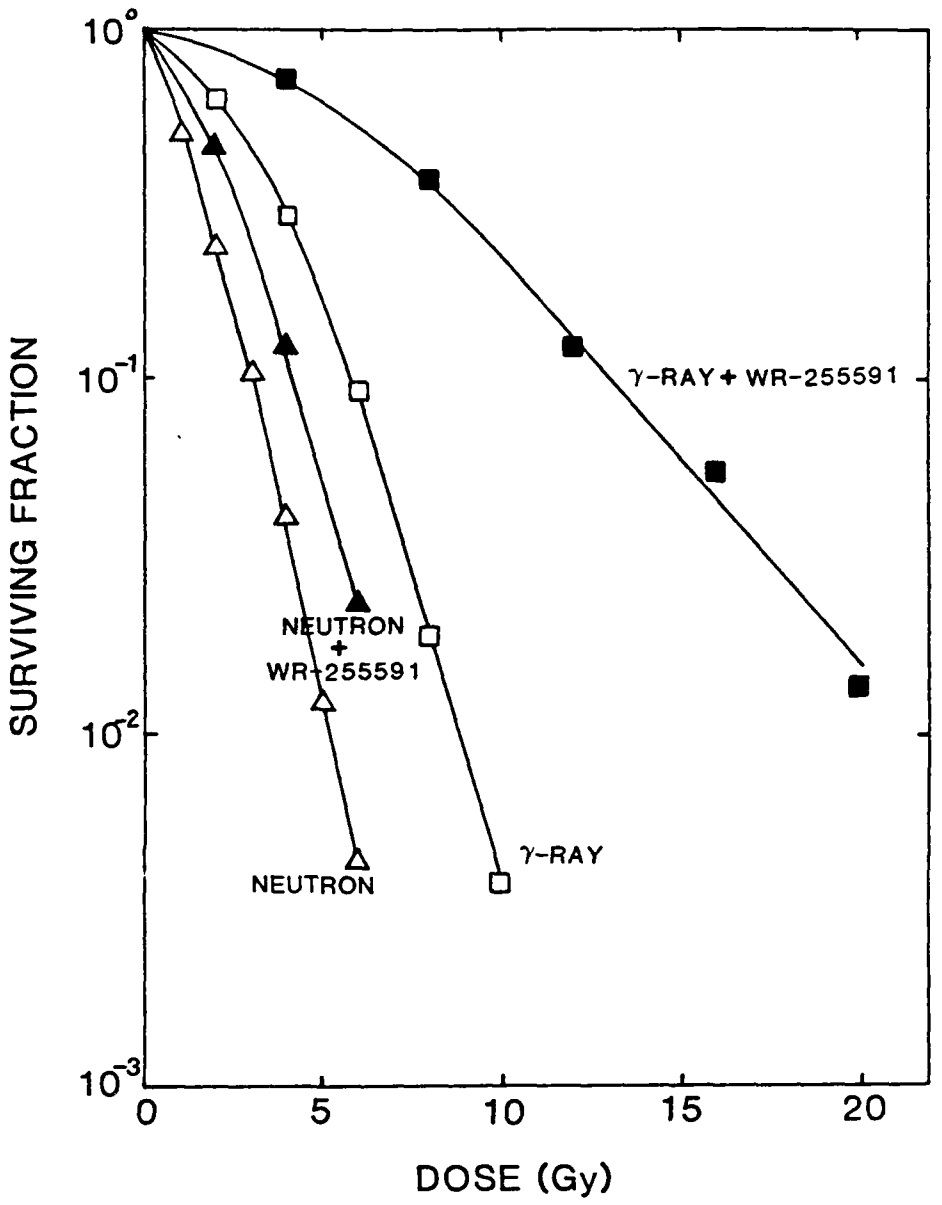


FIGURE 20. Effect of WR-255591 on the survival of neutron-irradiated CHO cells. The cells were irradiated with either 42-MeV neutrons (Δ, ▲) or ^{137}Cs-γ-rays (□, ■) with (closed symbols) or without (open symbols) a 30-min pretreatment with 6 mM WR-255591.

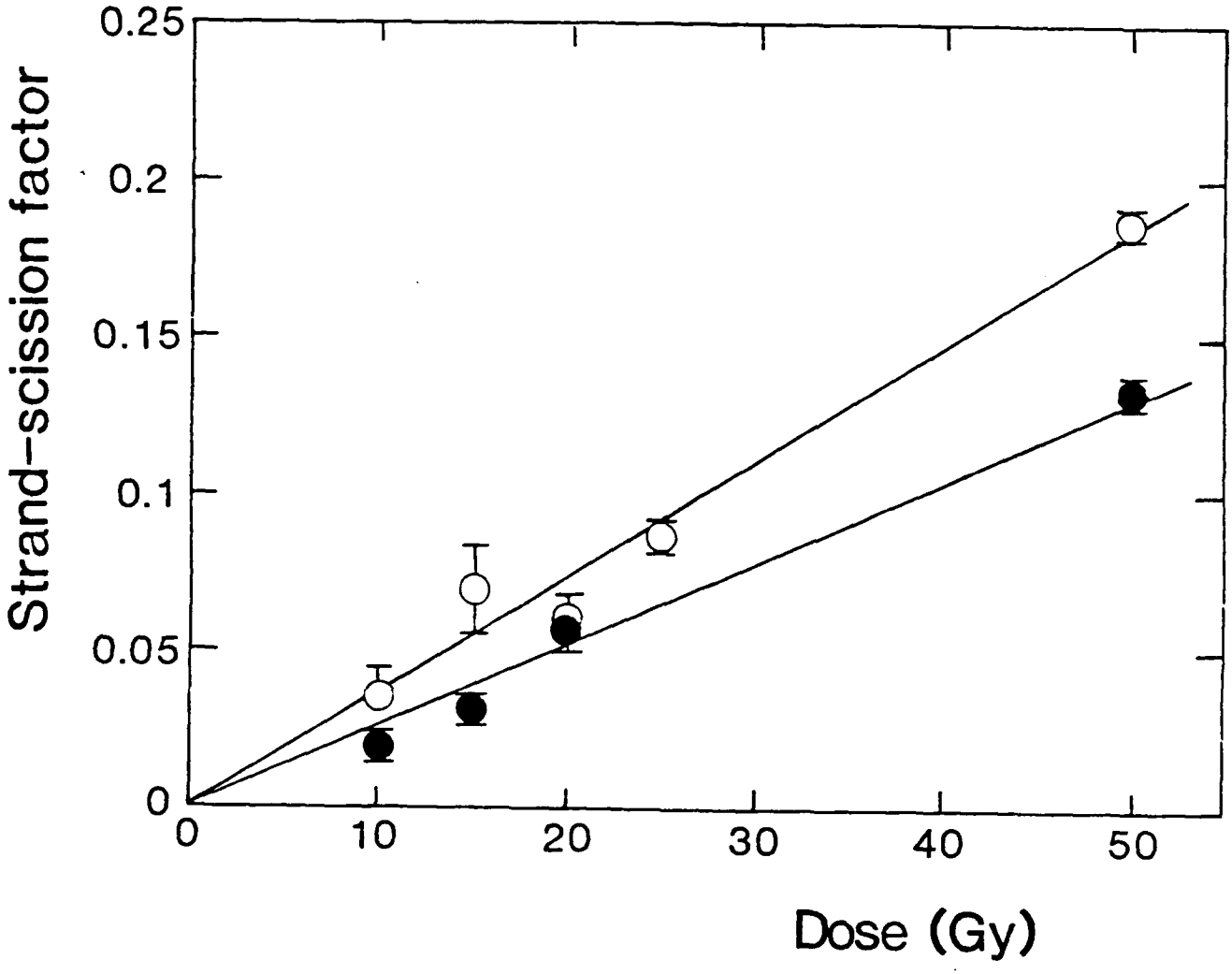


FIGURE 21. Effect of WR-255591 on the yield of neutron-induced DNA double-strand breaks. CHO cells were irradiated on ice either in growth medium (○) or following a 30-min incubation with 6 mM WR-255591 (●). The drug was present for 30-min prior to and throughout the duration of the irradiation. Double-strand breaks were assayed either at pH 7.0.

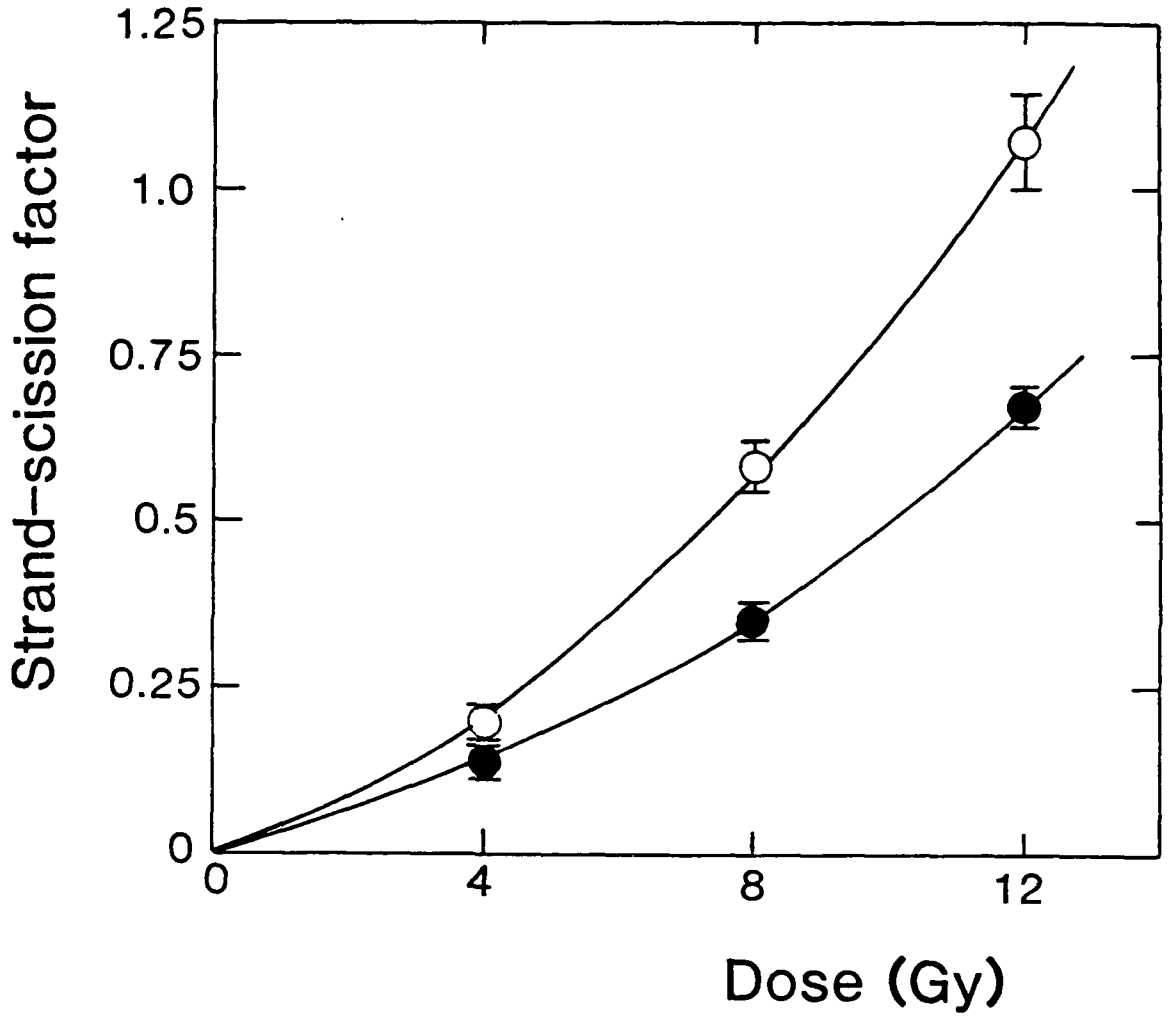


FIGURE 22. Effect of WR-255591 on the yield of neutron-induced DNA single-strand breaks. CHO cells were irradiated with on ice with 42-MeV neutrons either in growth medium (O) or following a 30-min treatment with 6 mM WR-151326 (●).

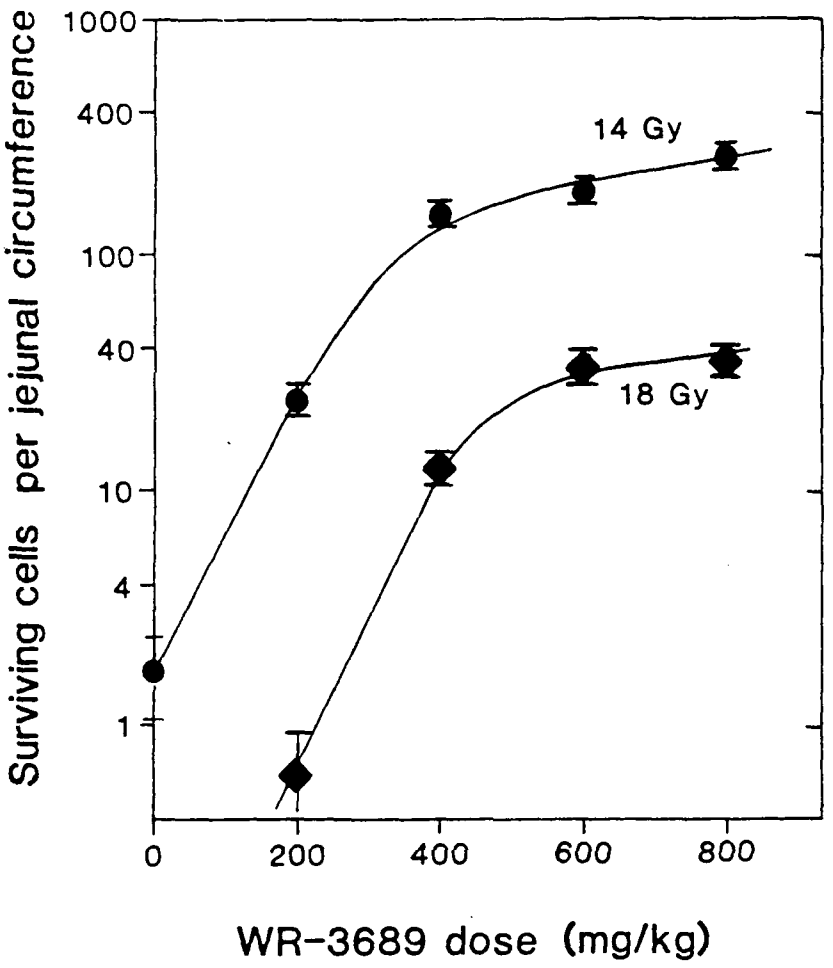


FIGURE 23. Dependence of jejunal crypt-cell survival on the dose of WR-3689. The drug was administered i.p. 30-min prior to irradiation with either 14 Gy (●) or 18 Gy (◆) of γ-rays.

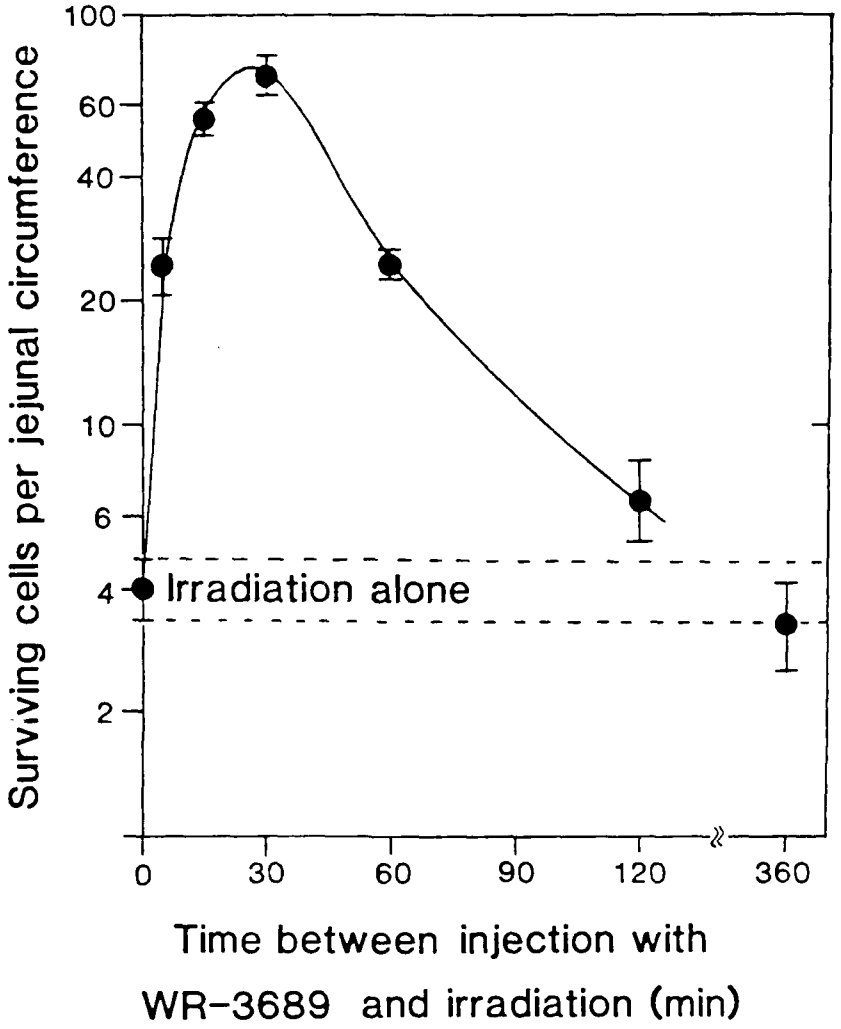


FIGURE 24. Dependence of jejunal crypt-cell survival on the timing of injection with WR-3689. The drug (200 mg/kg i.p.) was given at various times prior to irradiation with 14 Gy of γ-rays.

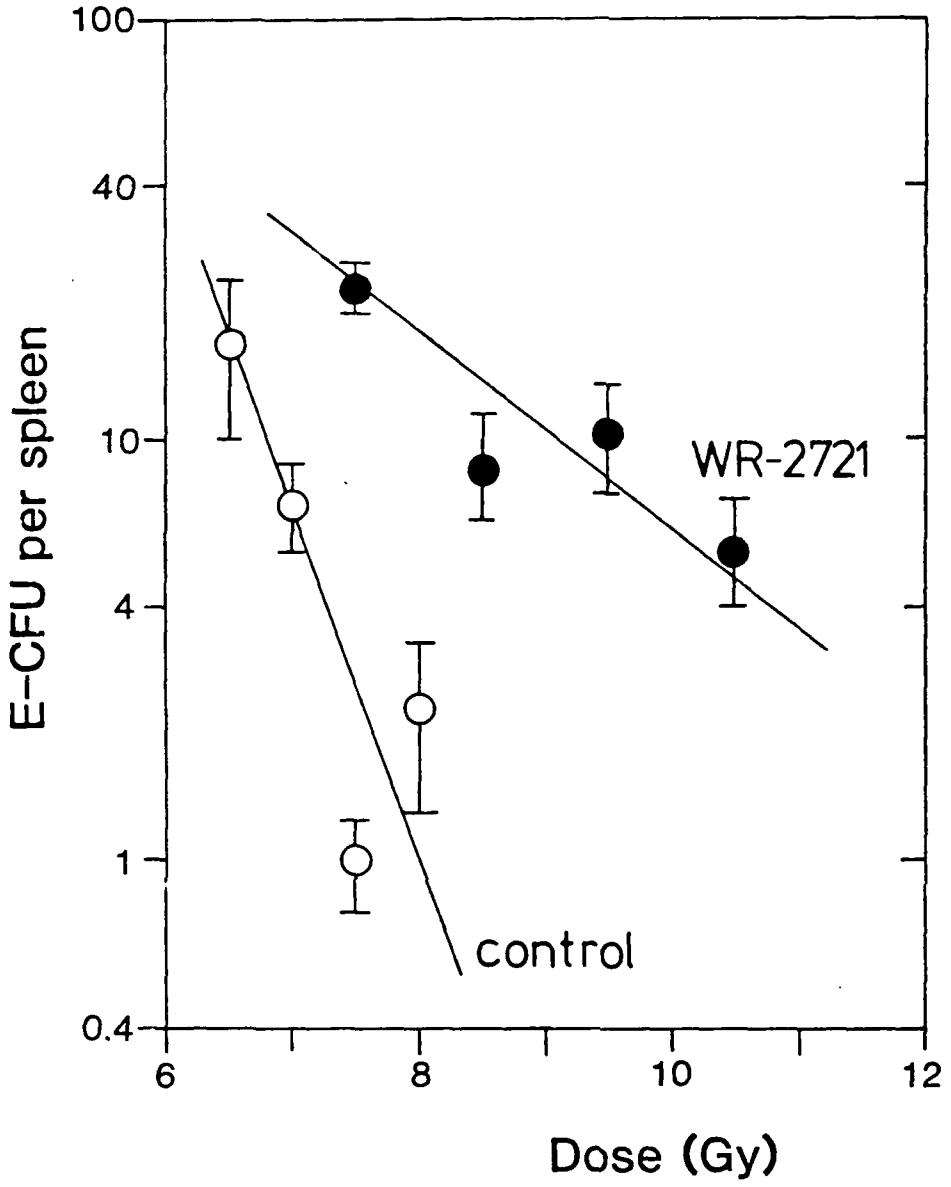


FIGURE 25. Effect of WR-2721 on the survival of mouse bone marrow cells. WR-2721 (400 mg/kg) was given i.p. 30-min before exposure to various doses of γ-rays. The spleen colony assay for endogenous colony-forming units (E-CFU) was used in this study.

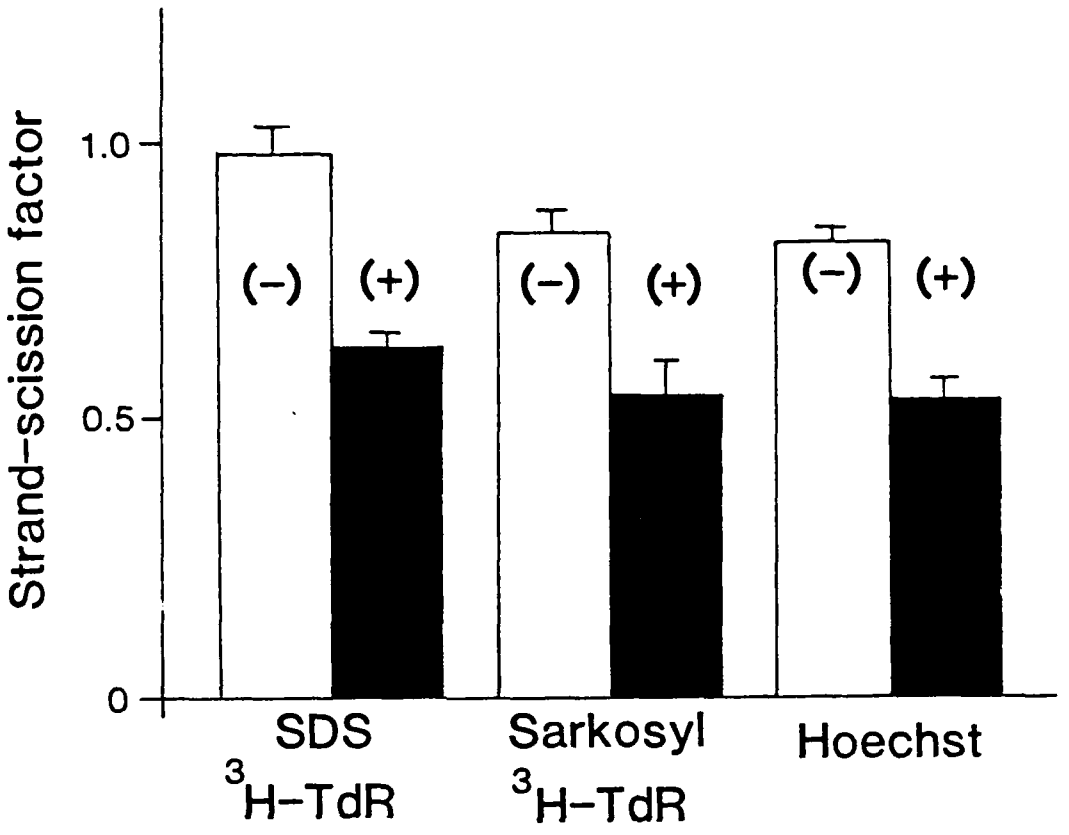


FIGURE 26. Effect of WR-2721 on the initial yield of γ-ray-induced DNA single-strand breaks in whole mouse bone marrow and in the proliferating bone marrow cells. In these experiments, the cells were lysed using either Sarkosyl or SDS-lysis. The ^{3}H-TdR assay characterizes the response of the proliferating cells while the Hoechst assay characterizes the entire tissue. The drug (400 mg/kg, i.p.) was given 30-min prior to whole-body irradiation with 10 Gy of γ-rays.

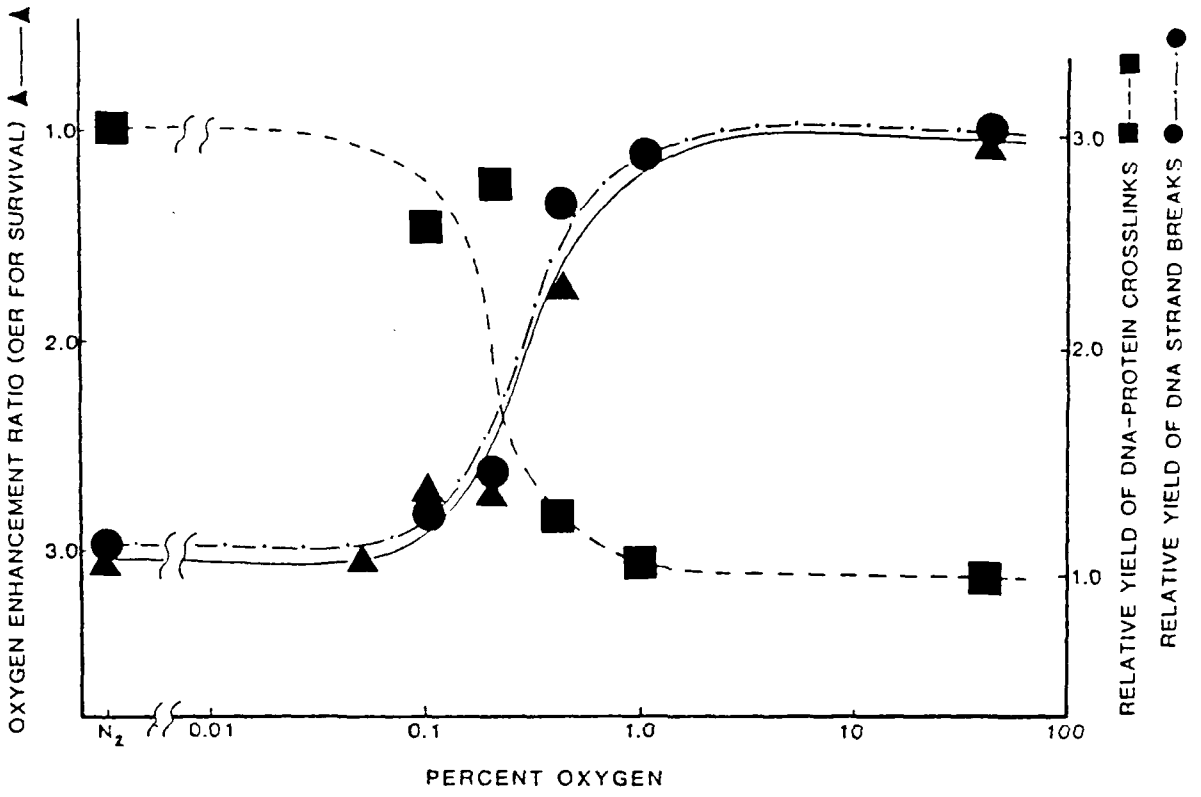


FIGURE 27. "K-curves" showing the effect of varying oxygen concentration on cell survival (▲) and on the relative yield of DNA single-strand breaks (●) and DNA-protein crosslinks (■) after irradiation of CHO cells with γ-radiation. The data are plotted as a function of the percent O_2 in the gassing mixture. The experimental equipment used in the acquisition of these data is shown in Figure 28. Single-cell suspensions were stirred at 4°C while being gassed with a mixture of 5% CO_2, varying concentrations of O_2, and a balance of N_2, for 3 h prior to irradiation. Cell survival was determined using a colony-forming assay; strand-breaks and DPCs were measured using alkaline elution.

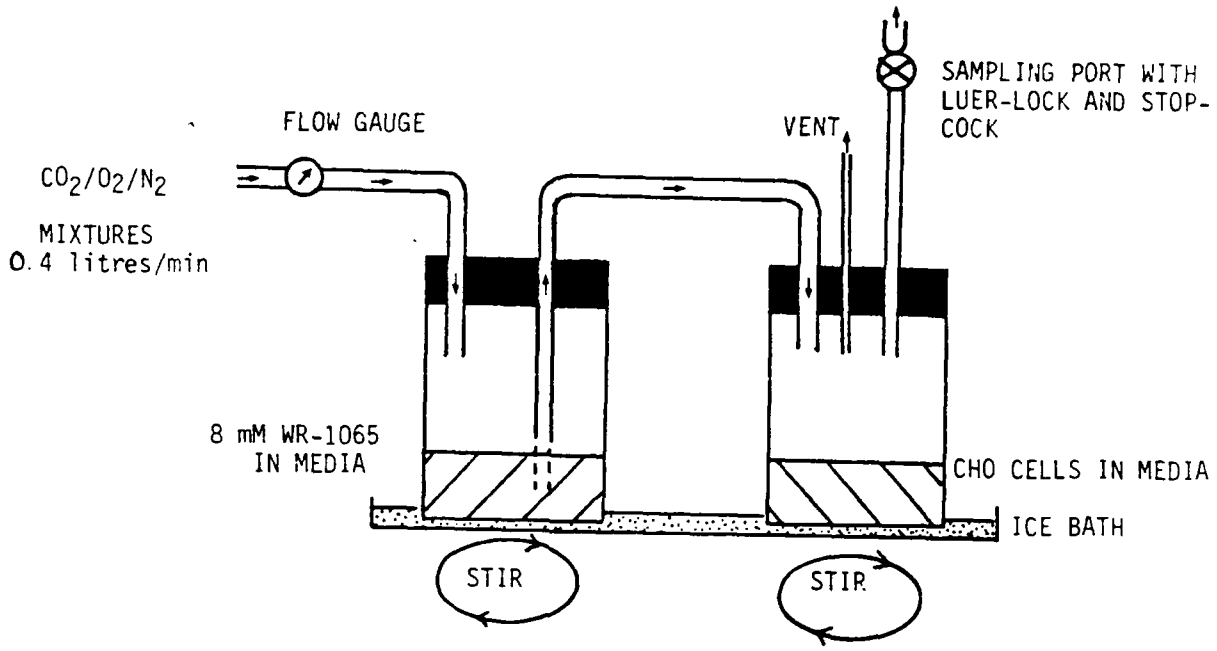


FIGURE 28. Diagram of the equipment used in our laboratory for measuring the dependence of radiobiological parameters on applied oxygen concentration. Actual oxygen tensions in the liquid phase are measured using a Clark-type electrode on an equivalent system but without CHO cells. The gases are specially mixed by the supplier to give an accuracy within 10 ppm of oxygen. A constant flow rate of 0.4 litres/min was maintained throughout. The drug and cells are mixed simply by lowering the arm (shown by the dotted line) into the drug-containing solution. Mixing an equal volume of cells and 8 mM drug solution gives a final drug concentration of 4 mM. The chamber containing the cells is then irradiated using a ^{137}Cs γ-ray source. Immediately after irradiation a sample of the cell/drug mixture is withdrawn through the sampling port using a syringe and used to determine either cell survival or DNA damage. No contamination of the cells with atmospheric oxygen results from this procedure, as evidenced by the measured OER value for survival which is in excess of 3.

Table 1: Structures of the Compounds Used in this Study.

WR-255591	$CH_3NH(CH_2)_3NHCH_2CH_2SH$
WR-3689	$CH_3NH(CH_2)_3NHCH_2CH_2SPO_3H_2$
WR-1065	$H_2N(CH_2)_3NHCH_2CH_2SH$
WR-2721	$H_2N(CH_2)_3NHCH_2CH_2SPO_3H_2$
Cysteamine (WR-347)	$HNHCH_2CH_2SH$
WR-151326	$CH_3NH(CH_2)_3NHCH_2CH_2CH_2SH$
WR-151327	$CH_3NH(CH_2)_3NHCH_2CH_2CH_2SPO_3H_2$
WR-168643	$[NaO_2S(CH_2)_4S]_2S$
DTT	$HSCH_2CH(OH)CH(OH)CH_2SH$

Table 2: Survival curve parameters for CHO cells treated with 4 mM WR-1065.

TREATMENT			MULTITARGET			LINEAR-QUADRATIC		
pre	γ	post	D_o	D_q	n	α	β	α/β
-	control	-	1.29 ±0.08*	2.89 ±0.55	9.39 ±1.71	0.183 ±0.023	0.0372 ±0.0029	4.92 ±0.73
-	4°C	60 min	1.19 ±0.09	2.85 ±0.57	10.8 ±2.1	0.186 ±0.033	0.0365 ±0.0038	5.10 ±1.03
30 min	4°C	-	2.49 ±0.27	5.30 ±0.61	8.42 ±0.32	0.100 ±0.016	0.0102 ±0.0012	9.78 ±1.97
30 min	37°C	-	2.72 ±0.14	5.25 ±0.94	6.91 ±1.19	0.085 ±0.010	0.0098 ±0.0008	8.69 ±1.28
30 min	37°C	60 min	2.63 ±0.17	5.52 ±1.19	8.17 ±1.68	0.095 ±0.012	0.0093 ±0.0009	10.2 ± 1.6

* ± s.e.m.

Table 3: Protection factors for cell killing and DNA strand-breakage in cultured CHO cells treated with radioprotective compounds prior to γ-irradiation.

Drug	Concentration	PF (survival)	PF (SSB)	PF (DSB)
WR-1065	4 mM	2.0 (37°C) 2.0 (0°C)	1.54 (37°C) 1.23 (0°C)	- 1.60 (0°C)
WR-255591	6 mM	2.3 (37°C) 2.3 (0°C)	1.40 (37°C) 1.31 (0°C)	- 1.83 (0°C)
WR-151326	6 mM	2.8 (37°C) -	1.78 (37°C) 1.68 (0°C)	- 2.80 (0°C)
cysteamine	10 mM	2.6 (37°C)* -	2.55 (0°C) 1.85 (37°C)	- -
	30 mM	3.3 (37°C)* -	3.84 (0°C) 2.58 (37°C)	- 4.24 (0°C)
DTT	25 mM	2.5 (37°C)*	2.26 (0°C) 2.10 (37°C)	- -
	50 mM	3.5 (37°C)* -	3.10 (37°C) -	- 3.23 (0°C)

*: determined using a single dose of 10 Gy and assuming dose-modification, therefore not a true protection factor.

Table 4: Protection factors for cell killing and DNA single-strand break (SSB) induction in tissues of mice treated with aminothiol radioprotectors prior to γ-irradiation.

Tissue	Drug	Dose* (mg/kg)	PF (survival)	PF (SSB)
Jejunum	WR-1065	200	2.0	1.13-1.28
	WR-2721	400	1.8	1.04-1.09
	WR-255591	300	2.1	-
	WR-3689	400	2.0	1.0
		800	2.5	1.0
Bone Marrow	WR-2721	200	2.3[a]	-
		400	1.5-2.2[b]	1.6

*: given i.p. 30 min before irradiation.
a: E. L. Travis: data obtained using exogenous spleen microcolony assay.
b: data obtained using endogenous spleen microcolony assay.

DISTRIBUTION LIST FOR ARMY ANNUAL REPORT

12 copies

Director
Walter Reed Army Institute of Research
Walter Reed Army Medical Center
ATTN: SGRD-UWZ-C
Washington, DC 20307-5100

1 copy

Commander
US Army Medical Research and Development Command
ATTN: SGRD-RMI-S
Fort Detrick, Frederick, Maryland 21701-5012

2 copies

Defense Technical Information enter (DTIC)
ATTN: DTIC-DDAC
Cameron Station
Alexandria, VA 22304-6145

1 copy

Dean
School of Medicine
Uniformed Services University of the
Health Sciences
4301 Jones Bridge Road
Bethesda, MD 20814-4799

1 copy

Commandant
Academy of Health Sciences, US Army
ATTN: AHS-CDM
Fort Sam Houston, Texas 78234-6100

END

DATE

FILMED

8-88

DTIC